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THE INFLUENCE OF YEAST EXTRACT AND JASMONIC ACID ON PHENOLIC ACIDS CONTENT OF *IN VITRO* HAIRY ROOT CULTURES OF *ORTHOSIPHON ARISTATUS*

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ABSTRACT
Phenolic acids represent a big group of plant secondary metabolites that can be used as food additives, nutraceuticals, and pharmaceuticals. Obtaining phenolic acids from the plant *in vitro* cultures provide an attractive alternative to produce high-value plant-derived products. The impact of yeast extract and jasmonic acid on the induction of defense responses and consequently the production of phenolic acids in *vitro* hairy root cultures of *O. aristatus* have been investigated. Treatment of *O. aristatus* cultures with jasmonic acid caused accumulation of 12.98 mg.g^{-1} DW of phenolic acids, elicitation with yeast extract resulted in the highest amount of phenolic acids, particularly in 17.99 mg.g^{-1} DW as compared to 4.03 mg.g^{-1} DW for the non-treated cultures. Individual phenolic acids showed a different response to elicitation. Particularly rosmarinic acid content on the control plot reached 2.89 mg.g^{-1} DW, while after the treatment with jasmonic acid is increased to 10.84 mg.g^{-1} DW and after yeast application, it was 14.31 mg.g^{-1} DW. Also, caffeic acid content increased until 0.75 and 2.01 mg.g^{-1} DW after application of jasmonic acid and yeast extract, while at the control plot its concentration was 0.58 mg.g^{-1} DW. Application of yeast extract influenced synthesis of phenolic acids *in vitro* cultures of *O. aristatus* stronger as jasmonic acid treatment.

Keywords: elicitors; yeast extract and jasmonic acid; phenolic acids; *in vitro* hairy root culture; *Orthosiphon aristatus*

INTRODUCTION
Due to the increased demand of consumers to obtain food, enriched with natural health-promoting components, the integrating of phenolic acids into the food matrix is an important task of food producers.

Phenolic acids are industrially relevant compounds, finding use and application in the food, pharmaceutical, cosmetic, and chemical industries. They are used as the raw material for the production of vanillin and preservatives, as a crosslinking agent for the preparation of food gels and edible films. It is expected that the market size of phenolic acids will grow exponentially by 2025, driven by increasing consumer inclination towards the health benefits of the products. For example, the natural ferulic acid market size was over USD 35 million in 2018, and the industry expects consumption at over 750 tons by 2025 **(Valanciene et al., 2020)**.

Phenolic acids are responsible for the adaptations of plants to environmental stresses because they are involved in various defensive and protective mechanisms against microorganisms, insects, and herbivores **(Vamanu and Nita, 2013)**. When infected by the pathogenic microorganism, plants respond with rapid activation of various spatially and temporally regulated defense reactions. These responses include oxidative cross-linking of cell wall proteins, production of phytoalexins, hydrolytic enzymes, and incrustation of cell wall proteins with phenolics, particularly phenolic acids **(Kim et al., 2001; Murthy et al., 2014)**.

Phenolic acids are synthesised from the amino acid phenylalanine in the shikimate pathway **(Ogata et al., 2004)**. In terms of conjugation with other metabolites in the plant, phenolic acids can be classified as free and bound phenolic acids **(Yang et al., 2008)**.

The application of phenolic acids, particularly chlorogenic, caffeic, and rosmarinic acid, influences the aroma and taste of food products **(Hunaefi et al., 2013)**. Thus, the characteristic aromas of cocoa and coffee, consisting of the mixture of chlorogenic and rosmarinic acids, have been produced by cell cultures of *Tlaeobroma cacao* and *Coffea arabica*, respectively **(Bais et al., 2002; Palianytsia et al., 2014)**. Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxy phenyl lactic acid**,** becomes one of the most essential phenolic substances as an ingredient of functional food and food supplements due to

its antioxidant activities **(Khadeer et al., 2011; Shetty, 2001)**.

Numerous plants are characterized by a high content of phenolic acids, among them *Orthosiphon aristatus*. This plant belongs to the *Lamiaceae* family and is important in Indonesian folk medicine **(Hunaefi and Smetanska, 2013)**. This plant has been reported to have high phenolic compounds in which rosmarinic acid is the most important and abundant one **(Mushtruk et al., 2020a)**. **Kumar and Gupta (2008)** indicated, that *O. aristatus* can be used for the development of functional foods, especially due to the high content of rosmarinic acid. However, mass cultivation of tropical *O. aristatus* is considered to be relatively difficult due to specific cultivation conditions.

Plant *in vitro* cultures can be used as a source of valuable secondary metabolites, which can further applied as food additives, nutraceuticals, and pharmaceuticals. The synthesis of phytochemicals by *in vitro* cultures in contrast to these in plants is not dependent on environmental conditions and quality fluctuations. In many cases, the chemical synthesis of metabolites is not possible or economically infeasible **(Smetanska, 2018)**.

Plant cell cultures have been proposed for the production of phenolic acids **(Petersen and Simmonds, 2003)**. *Lithospermum erythrorhizon* cell culture synthesized rosmarinic acid, which was not presented in plant tissues **(Szabo, Thelen and Petersen, 1999)**. It has been reported that cell cultures of *Coffea arabica* as well as of *Theobromo cacao* synthesized chlorogenic acid **(Kurata et al., 1998; Rao and Ravishankar, 2002)**.

However, in many cases *in vitro* hairy root cultures can be preferred as comparing to *in vitro* callus or cell suspension cultures for mass production of the targeted compound as they are more genetically stable and can possess a higher rate of biomass accumulation in comparison to cell cultures **(Cai, Kastell and Smetanska, 2014)**. *In vitro,* hairy root cultures of *Coleus blumei* were reported to accumulate rosmarinic acid **(Bauer, Kiseljak and Jelaska, 2009)**.

Plant *in vitro* cultures shows physiological and morphological responses to microbial, physical, or chemical factors, known as elicitors **(Kovalyshyna et al., 2020b)**. Elicitation is one of the most efficient strategies that lead to the intensification of the synthesis of many secondary metabolites in the plant *in vitro* cultures **(Georgiev, Eibl, and Zhong, 2013)**. Elicitors play an important role in the modulation of biosynthetic pathways and can be used as effective tools to stimulate the synthesis of plant secondary metabolites **(Kurata et al., 1998)**. Since phenolic acids protect plants from environmental changes, the way to induce their synthesis is to apply unfavorable factors for example to simulate pathogen or herbivores attack, osmotic or irradiation stress, etc. Biotic stress can be caused by biotic elicitors as yeast extracts, particularly cell wall fragments of *Saccharomyces cerevisiae* as well as jasmonic acid, which can be formed by the action of the plant on microbial cell walls, also methyl jasmonate, salicylic acid, and ethephon **(Hunaefi and Smetanska, 2013; Schreiner et al., 2011)**.

From the experimental test of elicitors and precursors on the synthesis of phenolic acid, jasmonic acid rapidly influenced the synthesis pathways of phenolic acids. This is most likely because jasmonic acid is reported to function as a signal transducer in plant cell cultures whereby it activates specific genes responsible for defense mechanisms resulting in higher production of almost all secondary metabolites within a short time-lapse. Jasmonic acid and its precursor, linolenic acid, are involved in the whole complex cascade between signal chain reactions of the elicitor-receptor complex and gene activation.

Ogata et al. (2004) reported that yeast extracts 5 $g.L^{-1}$ as an elicitor induced an increase of rosmarinic acid content in *Lithospermum erythrorhizon* cell suspension cultures of 4 fold compared to the group of control. Similarly, **Kim et al. (2001)** reported that the stimulation of rosmarinic acid biosynthesis in *Agastache rugosa* O. Kuntze in response to the addition of yeast extract could elevate the rosmarinic acid content up to 5.7-fold compared to non-elicited suspension cells. A more dramatic increase 7 fold of control in rosmarinic acid accumulation had reported in the same plant *O. aristatus* using a cell suspension culture system by **Sumaryono et al. (1991)**. **Hunaefi and Smetanska (2013)** reported that among several treatments with stress substances, elicitation with yeast extract resulted in the highest amount of total phenolic compounds, particularly in 324.5 $mg.g^{-1}$ DW (dry weight), in shoot cultures of *O. aristatus* as compared to 151.2 $mg.g^{-1}$ DW for the non-treated cultures. The content of rosmarinic acid in cultured cells of *Lithospermum erythrorhizon* increased after the addition of yeast extract: a maximum was reached in 24 h **(Petersen and Simmonds, 2003)**. When the plant cells were treated with yeast extract on the 6th day of the cultivation, the level of rosmarinic acid increased 2.5 times.

It has been reported that jasmonic acid and its methyl ester influenced the phenylpropanoid pathway by activating the enzyme phenylalanine ammonia-lyase in cell suspension cultures of soybean (*Glycine max*) **(Gueven and Knorr, 2011)**. Similar results were obtained in studies where jasmonic acid was used as an enhancer for the synthesis of phenolic compounds, particularly anthocyanins with a maximum production of phenolics on the 2nd day after application **(Curtin, Zhang, and Franco, 2003)**.

Therefore, in our research, we intended to prove the use of *in vitro* cultures *O. aristatus* as sustainable sources of phenolic acids through the cultivation of them under the influence of biotic elicitors as yeast extract and jasmonic acid.

Scientific hypothesis

The hypothesis of research work hinged on the assumptions that the synthesis of phenolic acids in *vitro* cultures could be positively influenced by the application of biotic elicitors, particularly jasmonic acid and yeast extract. As an object of the study *in vitro* hairy root culture of *Orthosiphon aristatus* has been selected due to the high content of valuable phenolic acids. According to obtained results the increase of the content of phenolic acids under the influence of elicitors, especially yeast extract, has been approved.

MATERIAL AND METHODOLOGY

Establishment and cultivation of *in vitro* hairy root cultures of *O. aristatus*

O. aristatus hairy root culture was established according to **Gabr et al. (2018)**. Seeds of *O. aristatus* were treaded with 70% ethanol for 20 – 30 seconds and then transferred

into 10% sodium hypochlorite solution for 5 min. Afterward, they were washed three times in sterilized bidestilled water and sewn on MS (Murashigi and Skoog) medium into 250 mL Erlenmeyer flasks. After 4 weeks sterilized plants were cut into 1 – 1.5 cm pieces (±100 mg fresh weight.L^{-1}) and placed in 250 mL Erlenmeyer flasks containing 50 mL liquid MS medium and *A. rhizogenes* suspension for 15 min and then transformed to MS medium. After 24 hours 500 mg.L^{-1} cefotaxime have been added to eliminate *A. rhizogenes*.

In vitro hairy root culture of *O. aristatus* was transferred to a new medium every four weeks and maintained at 25 ±2 °C on a rotary shaker at 100 rpm. The best line of *in vitro* culture was the third generation from the first establishment. This line has been used for represented research.

Treatment with elicitors

Liquid MS media were prepared as follows: 1) MS medium (Control); 2) MS medium and 100 μM jasmonic acid; 3). MS medium and 5 g.L^{-1} yeast extract.

Extraction of phenolic acids and identification by HPLC

20 mg of the powdered samples were extracted for 15 minutes with 750 μL of 70% methanol (VWR, Germany) in bi-distilled water in an ultrasonic water bath on ice. Afterward, the samples were centrifuged for 5 minutes at 13000 min^{-1} in Eppendorf centrifuge. The supernatants were collected and the pellets were re-extracted twice with 500 μL 70% methanol in bi-distilled. 40 μL of cinnamic acid (VWR, Germany) diluted in 70% methanol in bi-distilled was used as an internal standard. The collected supernatants from each sample were concentrated in a rotary evaporator (SPD 111V Speed Vac. Concentrator, Thermo Scientific, USA; CVC 3000V, Vacuubrand GmbH, Wertheim, Germany) at 25 °C under vacuum to remove the solvent completely. The residues were dissolved in 1 mL HPLC MilliQ water. The samples were filtered through 0.22 μM cellulose acetate filters (Corning® Costar® Spin-X® Plastic Centrifuge Tube Filters, Sigma-Aldrich Chemie GmBH, Steinheim, Germany). Analyzing of phenolic acids was performed on HPLC (UltiMate SR-3000, Dionex, Idstein, Germany), equipped with LPG-3400SD pump, WPS-3000SL automated sample injector, AcclaimPA C16-column (3 μm, 2.1 x 150 mm, Dionex) and DAD-3000 diode array detector (Dionex) and software Chromeleon 6.8. The column was operated at a temperature of 35 °C. The mobile phase consisted of 0.1% phosphoric acid (VWR, Germany) in HPLC water (eluent A) and 40% acetonitrile (VWR, Germany) in HPLC water (v/v, eluent B). A multistep gradient was used for all separations with an initial injection volume of 40 μL and a flow rate of 0.4 mL.min^{-1}. The multistep gradient was used as follows: 0 – 1 min: 0.5% B; 1 – 10 min: 0.5 – 40% B; 10 – 12 min: 40% B; 12 – 18 min: 40 – 80% B; 18 – 20 min: 80% B; 20 – 24 min: 80 – 99% B; 24 – 30 min: 99 – 100% B; 30 – 34 min: 100 – 0.5% B; 34 – 39 min: 0.5% B. Diode array detection was used for the identification of the compounds. Retention times and UV/visible absorption spectra of the peaks were compared with those of the authentic standards. The phenolic acid quantity was calculated from HPLC peak areas. The standard curves were obtained by plotting the peak area ratios (Y-axis) of standard compounds to the internal standard versus the concentrations of standard compound (X-axis). According to these standard curves, the linear regressions (Y = mX + b) and correlation coefficients as well as corrected with response factors were computed.

The concentration of phenolic acids was calculated using the following equation (1):

$$phenolic\ acid\ concentration\left[\mu mol\ g^{-1}DW\right] = \frac{AEPh \cdot RFPh \cdot cST}{AESt} \cdot \frac{V\,\mathrm{Pr}}{VInj} \cdot \frac{1}{PE} \quad (1)$$

Where:
AEPh – Peak area phenolic acid; RFPh – Response factor phenolic acid; cSt – Concentration internal standard (nmol); AESt – Peak area internal standard; VP – Volume after extraction (μL); VInj – Injection volume (μL); PE – Sample weight (mg).

Statistical Analysis

Experimental results concerning this study are reported as means ± standard deviation (*SD*). The data were subjected to analysis of variance (ANOVA) and the level of statistical significance was set at *p*-values <0.05 using SPSS version 17.0 (SPSS Inc. Chicago, IL, USA). Correlation analyses between the parameters were done using the "ProcCorr" procedure in SAS for Windows (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

The analysis of the growth dynamic of hairy roots of *O. aristatus* have shown, that the biomass increased from 3.1 g FW (fresh weight) $flack^{-1}$ on the 1st day of the experiment to 8.2 g FW $flack^{-1}$ on the 7th day, 11.4 g FW $flack^{-1}$ on the 10th day up to 150 g FW $flack^{-1}$ on the 14th day (Figure 1).

Application of jasmonic acid caused a decrease in fresh weight accumulation by 78% on the 14th day of the experiment as compared to the control plot, but treatment with yeast extract led to a 23% increase of the biomass toward the control.

To improve the production of phenolic compounds in *vitro* hairy root cultures, supplementation of elicitation with yeast extract and jasmonic acid has been used **(Cai et al., 2014)**. However, previously there was no report about the influence of these elicitors on the synthesis of phenolic acids *in vitro* hairy root cultures of *O. aristatus*.

The analysis of total phenolic acid in *vitro* cultures of *O. aristatus* have shown that during the cultivation time of 14 days hairy roots of untreated hairy roots accumulated 1.35 mg.g^{-1} DW of the substances at the beginning and 4,03 mg.g^{-1} DW by the end of the experiment (Figure 2). Treated with elicitors cultures answered during the first 24 hours after application, however, jasmonic acid caused a 3-times decrease in phenolic acid content as compared to the untreated hairy roots. In contrast, yeast extract application caused stimulation of the synthesis of phenolic acids by almost 2.5-times as compared to the control. The positive effect of the treatment with jasmonic acid has been observed between the 3rd and 7th days of the experiment when the content of phenolic acids increased from 1.11 to 9.30 mg.g^{-1} DW. Yeast extract application influenced

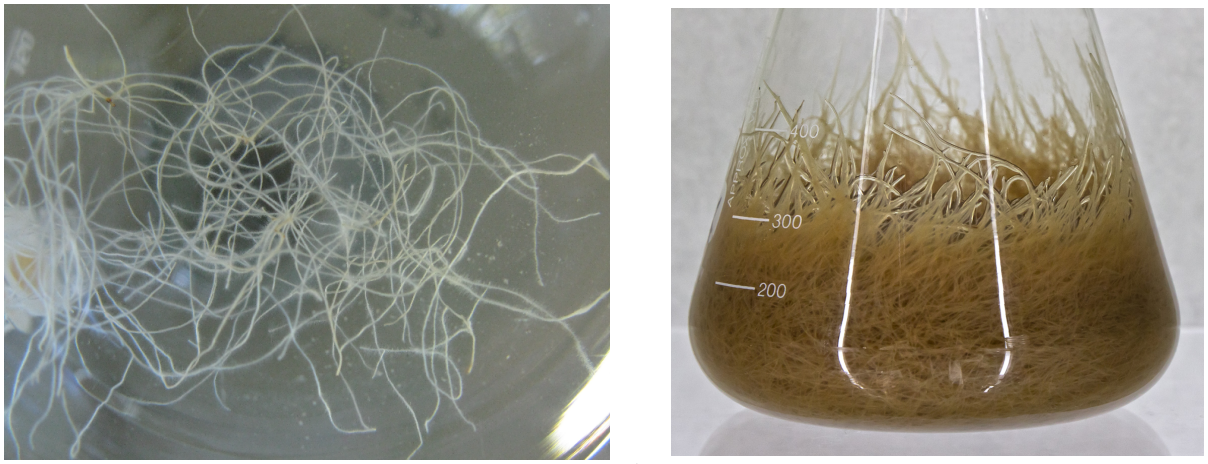


Figure 1 Hairy root culture of *O. aristatus* on the 1st and 14th day of experiment.

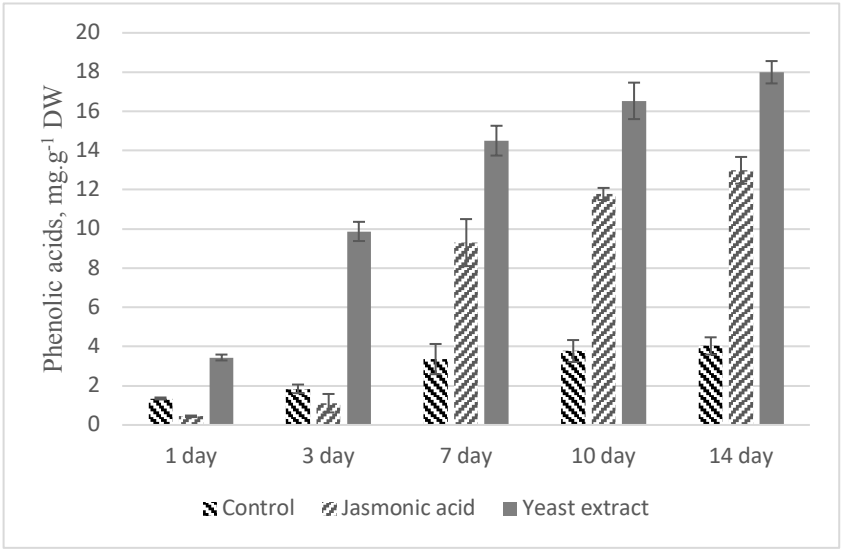


Figure 2 Impact of elicitors on the content of phenolic acids in *in vitro* hairy root cultures of *O. aristatus.*

positively the process of phenolic acid synthesis during the first 7 days after application, afterwards, the intensity of increment of phenolic acid accumulation became slower.

By the end of the experiment, both elicitors caused the rising of total phenolic acid, particularly jasmonic acid 3.2-times, and yeast extract 4.5-times more in comparison to the control plot.

Authors of scientific works **(Yazaki, 2017; Izuishi et al., 2020; Sheiko et al., 2019)** reported that yeast extracts added in a quantity of 5 $g.L^{-1}$ into the cultivation media of *Lithospermum erythrorhizon* cell suspension cultures induced an increase in phenolic acid content by 4-folds as compared to the control. Authors of scientific works **(Guo et al., 2020; Kovalyshyna et al., 2020a)** reported that the stimulation of rosmarinic acid biosynthesis in *Agastache rugosa* O. Kuntze in response to the application of yeast extract elevated phenolic acid content up to 5.7-folds in comparison to non-elicited suspension cell cultures. A more dramatic increase in phenolic acid accumulation, particularly 7-fold of control, had been reported in the cell suspension culture of *O. aristatus* after yeast extract treatment **(Sumaryono et al., 1991; Kasem, 2018)**. The content of rosmarinic acid in cultured cells of *Lithospermum erythrorhizon* increased after the addition of yeast extract **(Kumar and Gupta, 2008)**. When the plant cells were treated with yeast extract on the 6th day of the cultivation, the level of rosmarinic acid increased 2.5 times.

Treatment with yeast extract stimulated synthesis of anthraquinones in cell cultures of *Morinda citrifolia* **(Doernenburg and Knorr, 1997)**, rosmarinic acid in *Coleus blumei* **(Swamy et al., 2018)**, flavonoids in *Glycyrrhiza uralensis* **(Zhang et al., 2009)**, isoflavonoids in *Pueraria candollei* **(Udomsuk et al., 2011)**.

Increasing of isoflavonoid biosynthesis by 5 – 6 times was observed by jasmonic acid addition to soy plant callus suspension culture **(Gueven and Knorr, 2011; Palamarchuk et al., 2019)**.

Analyzing individual phenolic acids in *vitro* hairy root culture of *O. aristatus* has shown the presence of vanillic, chlorogenic, caffeic, p-coumaric, sinapic, and rosmarinic acids (Figure 3). Rosmarinic acid was the major one, followed by caffeic and vanillic acids. Rosmarinic acid content on the control plot reached 2.89 $mg.g^{-1}$ DW, while after the treatment with jasmonic acid it increased to 10.84 $mg.g^{-1}$ DW and after yeast application, it was 14.31 $mg.g^{-1}$ DW (Table 1).

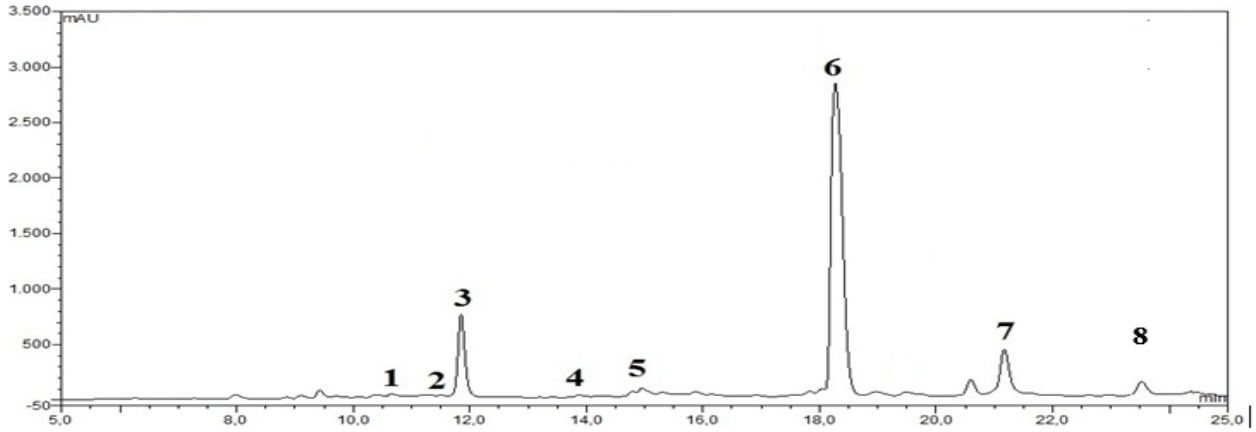
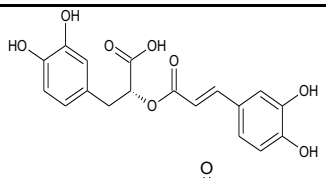
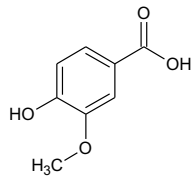
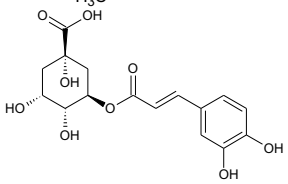
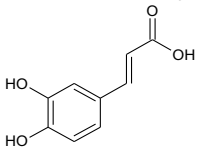
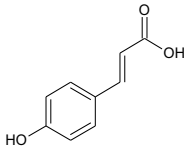
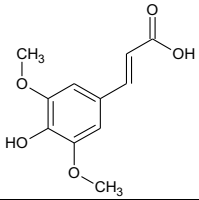


Figure 3 HPLC chromatogram of *in vitro* culture of *O. aristatus.* Note: 1 – vanillic acid; 2 – chlorogenic acid; 3 – caffeic acid; 4 – p-coumaric acid; 5 – sinapic acid; 6 – rosmarinic acid; 7 – cinnamic acid.

Table 1 The individual response of phenolic acids of *in vitro* culture of *O. aristatus* subjected to different treatments.

Phenolic acids	Chemical structure	Control, mg.g^{-1} DW	Jasmonic acid		Yeast extract	
			mg.g^{-1} DW	Fold of control	mg.g^{-1} DW	Fold of control
rosmarinic acid		2.89 ± 0.36	10.84 ± 0.95	3.75	14.31 ± 0.78	4.95
vanillic acid		0.31 ± 0.03	0.87 ± 0.11	2.80	1.15 ± 0.14	3.72
chlorogenic acid		0.07 ± 0.01	0.08 ± 0.01	1.10	0.13 ± 0.02	1.90
caffeic acid		0.58 ± 0.02	0.75 ± 0.08	1.30	2,01 ± 0.13	3.46
p-coumaric acid		0.04 ± 0.01	0.10 ± 0.02	2.40	0.14 ± 0.02	3.50
sinapic acid		0.14 ± 0.02	0.35 ± 0.04	2.5	0.25 ± 0.02	1.82

The basal level of vanillic acid (control) was 0.031 mg.g^{-1} DW, by elicitation with jasmonic acid its content was found higher by 2.8-fold while in the yeast treated culture vanillic acid content was 3.72-fold of the level of control.

The highest concentrations of caffeic and coumaric acids produced by *in vitro* culture of *O. aristatus* were elicited with yeast extract, which was 3.46 and 3.50-fold higher compared to the control level.

The results corresponded with previous investigators using elicitors for producing phenolic acids, particularly rosmarinic acid, in different plants. Elicitors had proven to induced significantly rosmarinic acid production in sprout

cultures of *O. aristatus* **(Hunaefi et al., 2018; Chua et al., 2018)** and cell cultures of *Lithospermum erythrorhizon* **(Ogata et al., 2004; Peng et al., 2019)**. Since in plants, phenolic acids are supposed to act as a preformed constitutively accumulated defense compound and therefore most of the successful cases reports in enhancement rosmarinic acid were by elicitors treatments **(Debnath et al., 2011; Mushtruk et al., 2020b)**.

Induction of rosmarinic acid accumulation in *O. aristatus* cell culture by yeast extract was not limited to cells in the lag phase but could also be provoked when cells were in the growth or stationary phase **(Castro et al., 2017)**. Authors of scientific works **(Bober et al., 2020; Zheplinska et al., 2019; Schulenburg et al., 2016)** reported that phenolic acid accumulation by precursors supplementation had reached a maximum at day 15 on *in vitro* culture of *Oregano*.

Overall, among all treatment yeast extract and jasmonic acid elicitation resulted in higher accumulation for phenolic acids as compared to the control. Phenolic acids were more responsive to yeast extract as jasmonic acid treatment.

These observations show how, in a single system, different phenolic acids of secondary metabolite products can show distinct differences in their responses

CONCLUSION

The aim of the represented research work was to establish the hairy root culture of *O. aristatus* and to develop the technology of its cultivation for obtaining valuable phenolic acids. The analysis of the growth dynamic of hairy roots of *O. aristatus* has shown, that during the cultivation on MS medium the biomass increased during 14 days of cultivation from 3.1 g until 15.0 g FW flack^{-1} on the 14th day. Application of jasmonic acid caused a decrease in fresh weight accumulation by 78% on the 14th day of the experiment as compared to the control plot, but treatment with yeast extract led to a 23% increase of the biomass toward the control.

The analysis of total phenolic acid in *vitro* cultures of *O. aristatus* has shown that during the cultivation time of 14 days hairy roots of untreated hairy roots accumulated 1.35 mg.g^{-1} DW of the substances at the beginning and 4.03 mg.g^{-1} DW by the end of the experiment. Treated with elicitors cultures answered during the first 24 hours after application, however, jasmonic acid caused a 3-times decrease in phenolic acid content and yeast extract application caused stimulation of the synthesis by 2.5-times as compared to the control. The positive effect of the treatment with jasmonic acid has been observed between the 3rd and 7th days of the experiment, but of the yeast extract application during the first 7 days after application. By the end of the experiment, both elicitors caused the rising of total phenolic acid, particularly jasmonic acid 3.2-times, and yeast extract 4.5-times more in comparison to the control plot.

Among all phenolic acids in the hairy root cultures, rosmarinic acid was the major one, followed by caffeic and vanillic acids. Its content on the control plot reached 2.89 mg.g^{-1} DW, while after the treatment with jasmonic acid is increased to 10.84 mg.g^{-1} DW and after yeast application, it was 14.31 mg.g^{-1} DW.

It is possible to conclude, that the hairy root culture of *O. aristatus* can be used for obtaining phenolic acids. The technology of *in vitro* cultivation can be optimized through the application of elicitors as jasmonic acid and yeast extract. However, the stimulation of the synthesis of phenolic acids after the application of yeast extract is more promising.

For further investigation of the proper use of elicitors, it would be advisable to investigate the application of elicitors during different stages of culture growth.

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Conflict of Interest:

The authors declare no conflict of interest.

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