New Synthetic Pyrazine Carboxamide Derivatives as Potential Elicitors in Production of Secondary Metabolite in In vitro Cultures

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ABSTRACT
Background: Silymarin, an active polyphenolic fraction of Silybum marianum, and high flavonoid content of Fagopyrum possess various interesting biological activities. The substituted pyrazine-2-carboxamides were previously used as effective elicitors of studied secondary metabolites.

Objective: To study the effect of new synthetic pyrazine carboxamide derivatives, N-(4-chlorobenzyl)-5-tert-butylpyrazine-2-carboxamide (1) and 3-(3-(trifluoromethyl) benzyl) amino pyrazine-2-carboxamide (2), on flavonolignans and flavonoid production in S. marianum and Fagopyrum esculentum in vitro cultures.

Materials and Methods: Callus and suspension cultures were cultured on MS medium containing α-naphthaleiacetic acid or 2,4-D. Three elicitor concentrations for different exposure times were tested. Dried and powdered samples of callus and suspension cultures were extracted with methanol and analyzed by DAD-HPLC.

Results: Compound 1 showed as a good elicitor of taxifolin production. The effect on silymarin complex was less visible with a maximum between 24 and 48 h after 3.292 × 10⁻⁴ mol/L concentration. The detailed analysis showed that silychristin was the most abundant. Compound 2 was effective in rutin production only in callus culture with maximum 24 h and 168 h after application of 3.3756 × 10⁻³ mol/L concentration and 48 and 72 h after 3.3756 × 10⁻⁴ mol/L concentration. Conclusion: From the results of the performed experiments, it can be concluded that compound 1 shows to be suitable elicitor for enhanced production of taxifolin and silychristin in S. marianum, mainly when 3.292 × 10⁻⁴ mol/L concentration was used, and compound 2 is suitable for increase rutin production in callus cultures and less appropriate for suspension cultures of F. esculentum.

Key words: Fagopyrum, flavonoids, flavonolignans, pyrazine carboxamide, Silybum

SUMMARY
• The influence of two new synthetic pyrazine-2-carboxamides derivatives on secondary metabolite content of Silybum marianum and Fagopyrum esculentum in vitro cultures was tested.

INTRODUCTION
Silymarin, an active polyphenolic fraction of Silybum marianum, is an ancient herbal remedy used to treat a range of liver disorders, including hepatitis, cirrhosis, and as a hepatoprotective nutritional supplement against poisoning from alcohol, chemical, wild mushroom, and environmental toxins.[i] Silymarin consists of a group of flavonolignans, namely silydianin, silychristin, silybin, and isosilybin. There are evidence that silymarin possesses also other interesting activities, e.g., anti-cancer, anti-inflammatory,[ii,iii] neuroactive, and neuroprotective.[iv] Its anti-oxidant activity is due to its free radical scavenging property, an increase in superoxide dismutase activity[v] and the inhibition of lipid peroxide formation.[vi]

Fagopyrum has been introduced in many countries as a food supplement. Among its nutrition values, it has attracted increasing attention for its notable anti-oxidant, hypocholesterolemic, anti-diabetic, anti-microbial, and anti-tumor activities; and is benefical for human health.[vii,vi] Many of health benefits have been attributed to high levels of phenolic compounds with antioxidant activity. Besides high rutin content, Fagopyrum also contains other flavonoids, such as quercetin, epicatechin, orientin, isoorientin, vitexin and isovitexin.[viii]
Nowadays, bioactive phytochemicals have become one of the major research topics. In vitro cultures derived from medical important species could be an alternative source of the bioactive metabolites. Several studies have shown that metabolite production in in vitro cultures was lower than in intact plants. The need to develop appropriate and the effective strategies for enhanced production of useful metabolites without gene modification has become an object of many studies. An increased synthesis of many secondary metabolites is usually the result of plant defense mechanisms to stress signals. Using suitable biotic or abiotic elicitors makes elicitation one of the most effective strategies for improving the yield of secondary metabolites production by in vitro cultures.[16,17] The most common and effective elicitors of flavonolignans and flavonoids used in previous studies include such as yeast extracts,[12,13] methyl jasmonate,[14,15] salicylic acid,[16] or heavy metals.[17]

The pyrazine ring is a part of many polycyclic compounds of biological and/or industrial significance. The widespread occurrence of pyrazines in nature, especially in the flavors of many food systems, their effectiveness at very low concentrations is responsible for the high interest in these compounds. Some substituted pyrazine carboxamides, tested in vitro, showed not only anti-mycobacterial and anti-fungal activities,[18,19] but also may play a role as herbicidal chemical agents due to their inhibition properties in photosynthetic electron transport in photosystem 2.[20] In previous works, the substituted pyrazine-2-carboxamides were used as effective elicitors enhancing not only the flavonolignan production in S. marianum cultures,[21] but also flavonoid production in Ononis arvensis cultures.[22]

On the basis of the above-mentioned results, the elicitor activity of two newly pyrazine derivatives [Figure 1] prepared by the Department of Pharmaceutical Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy in Hradec Králové was evaluated. Time and concentration dependent manner for stimulation of flavonolignan and flavonoid production in callus and suspension cultures of S. marianum and F. esculentum was chosen.

**MATERIALS AND METHODS**

**General**

All organic solvents used for the synthesis were of analytical grade. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). The amino dehalogenation reaction was performed in a CEM Discover microwave reactor with a focused field connected to an Explorer 24 autosampler (CEM Corporation, Matthews, NC, USA), and this equipment was running under CEM’s Synergy™ software for setting and monitoring the conditions of reactions. The temperature of the reaction mixture was monitored by the internal infrared sensor. The progress of the reactions was checked by using Merck Silica 60 F254 TLC plates (Merck, Darmstadt, Germany). Compounds were purified d by using an automated chromatograph CombiFlash R (Teledyne Isco, Lincoln, NE, USA) using columns filled with Kieselgel 60, 0.040–0.063 mm (Merck, Darmstadt, Germany); gradient elution (hexane/ethyl acetate), detection wavelength 260 nm, and monitor wavelength 280 nm. NMR analysis was performed on Varian Mercury VX-BB 300 (Varian, Palo Alto, CA, USA) at 300 MHz for H and 75 MHz for C. Chemical shifts were recorded as δ values in parts per million (ppm) and were indirectly referenced to tetramethylsilane. IR spectra were recorded in KBr blocks on Nicolet Impact 400 (Nicolet, Madison, WI, USA). The elementary analysis was performed on CE Instruments EA-1110 CHN analyzer (CE Instruments, Wigan, UK). Melting points were determined on Stuart SMP30 melting point apparatus (Bibby Scientific limited, Staffordshire, UK) and are uncorrected.

**Synthesis of pyrazine elicitors 1 and 2**

N-(4-chlorobenzyl)-5-tet butylpyrazine-2-carboxamide (1) was synthesized through aminolysis of 5-tet-butylpyrazinecarboxylic acid chlorides with 4-chlorobenzylamine.[9] The second elicitor, 3-(3-(trifluoromethylbenzyl) amino) pyrazine-2-carboxamide (2) was prepared through amino dehalogenation of 3-chloropyrazine-2-carboxamide with 3-trifluoromethylbenzylamine and performed in microwave reactor (140°C, 30 min, 120 W, methanol as a solvent and pyridine as a base).[23]

The N-(4-chlorobenzyl)-5-tet-butylpyrazine-2-carboxamide (1) White crystalline compound. Yield: 83%; m.p. 95.1–96.5°C; 1H-NMR (CDCl3) δ 9.32 (s, 1H, H3), 8.54 (s, 1H, H6), 8.10 (s, 1H, NH), 7.34–7.25 (4H, m, ArH), 4.63 (2H, d, J = 6.2 Hz, CH2), 1.41 (9H, t, J = 6.0 Hz, tert-butyl H); 13C-NMR (CDCl3) δ 167.5, 163.4, 142.8, 141.2, 139.1, 136.5, 133.4, 129.1, 128.8, 42.6, 37.0, 29.7 IR (cm−1) 3331 (N-H), 1657 (C = O); Anal. Calcd. for C13H11F3N4O; 63.26% C, 5.97% H, 13.83% N; Found: 63.19% C, 6.01% H, 13.77% N.

The 3-(3-(Trifluoromethyl benzyl) amino) pyrazine-2-carboxamide (2). White crystalline compound. Yield: 35%; m.p. 98.1–100.3°C; 1H-NMR (CDCl3) δ 9.23 (1H, t, J = 6.1 Hz, NH), 8.22 (1H, d, J = 2.2 Hz, H6), 8.19 (1H, bs, NH), 7.80 (1H, d, J = 2.2 Hz, H5), 7.72 (1H, bs, NH), 7.66 (1H, s, H2), 7.64–7.50 (5H, m, H4, H5, H6), 4.74 (2H, d, J = 6.1 Hz, CH2). 13C-NMR (CDCl3) δ 168.9, 154.2, 146.4, 141.6, 131.4, 130.5, 129.6, 129.2 (q, J = 31.3 Hz), 127.0, 124.4 (q, J = 27.0 Hz), 123.8 (q, J = 3.9 Hz), 123.7 (q, J = 6.1 Hz), 43.1; IR (cm−1) 3468 (NH), 1674 (C = O); Analytical. Calcd. for C13H11F3N4O (296.25): 52.71% C, 3.74% H, 18.91% N; Found: 52.58% C, 3.61% H, 18.82% N.

**Plant material**

Callus cultures were derived from the germing seeds of plant S. marianum (L.) Gaertn (Asteraceae) and Fagopyrum esculentum L. (Polygonaceae). S. marianum in vitro cultures in the 64th–69th passages were used. Calluses were cultured on MS0.1 medium containing α-naphthaleneacetic acid as growth regulator at a concentration of 5.4 × 10−5 mol/L. Callus cultures were cultivated on paper bridges in Erlenmeyer flasks and suspension cultures in 250 mL growth flasks with shaking at 120 rpm in growth chambers at 25°C under a 16 h photoperiod. F. esculentum in vitro cultures in the 22nd–24th passages were used. Calluses were cultured on MS medium containing 2.4-dichlorophenoxyacetic acid (2,4-D) as growth regulator at a concentration of 4.5 × 10−5 mol/L. Callus cultures were cultivated on paper bridges in Erlenmeyer flasks and suspension cultures in 100 mL growth flasks with shaking at 150 rpm. Growth chamber parameters were the same as above mentioned conditions.

**Elicitor application**

As elicitors, N-(4-chlorobenzyl)-5-tet-butyl-pyrazine-2-carboxamide (compound 1) at concentrations 3.292 × 10−5 mol/L (c1); 3.292 × 10−4 mol/L (c2), 3.292 × 10−3 mol/L (c3), and 3-(3-(trifluoromethyl benzyl) amino) pyrazine-2-carboxamide (compound 2) at concentrations 3.3756 × 10−5 mol/L (c4), 3.3756 × 10−4 mol/L (c5), 3.3756 × 10−3 mol/L (c6), were used. Elicitors were dissolved in 96% ethanol, and 1 mL of solution was used.
added to the nutrient medium. Compound 1 was added to *S. marianum* callus culture on the 25th day and to the suspension on the 17th day of cultivation. Compound 2 was added to *F. esculentum* callus culture on the 28th day and to the suspension on the 17th day of cultivation. For each concentration of elicitors, 32 flasks were used. For determination of flavonolignan and flavonoid contents, samples were harvested at six different time points after elicitation; 6, 12, 24, 48, 72, and 168 h. Simultaneously, the controls (without elicitors) were run for 24 and 168 h.

### Analysis of flavonolignans

Dried and powdered samples of callus tissue and suspension culture cells were extracted twice (in a water bath under reflux cooler) with 80% (v/v) methanol for 10 min. The samples of nutrient media were evaporated on a water bath to dryness and then dissolved in 80% (v/v) methanol. The extracts and nutrient media samples were filtered through a 0.45 μm microfilter and approximately 1.7 mL of filtrate was analyzed by HPLC with DAD detection. HPLC conditions and standard preparation were the same as described previously in.[21]

### Analysis of flavonoids

Dried and powdered samples of callus tissue and suspension culture cells were extracted twice (in a water bath under reflux cooler) with 80% (v/v) methanol for 30 min at 60°C. The extracts were filtered, 5 mL of 80% (v/v) methanol was added and then sonicated for 15 min. The samples of nutrient media were evaporated on a water bath to dryness and then dissolved in 80% (v/v) methanol. The extracts and nutrient media samples were filtered through a 0.20 μm microfilter, and approximately 1.7 mL of filtrate was analyzed by HPLC with DAD detection. The HPLC method was modified at the department according to the Czech Pharmacopoeia.[22]

### Statistical analysis

All analyses were carried out in a minimum of three independent samples for each elicitation period and each concentration of elicitor. To determine differences between values of samples, the t-test was used. The values of *P* ≤ 0.05 were considered as significantly different.

### RESULTS AND DISCUSSION

**Elicitation with N-(4-chlorobenzyl)-5-tert-butyl-pyrazine-2-carboxamide (1)**

The results outline in Tables 1 and 2 indicate that compounds 1 affected predominantly the taxifolin production in callus and suspension cultures of *S. marianum*.

### Callus cultures

The maximum values of taxifolin were detected 6 h (1.419 mg/g DW) and 24 h (1.761 mg/g DW) after elicitation with a concentration of c<sub>1a</sub>. The lowest production of taxifolin was monitored, where the highest concentration of elicitor was used. The best elicitation effect in callus culture was found 24 h after treatment with c<sub>1a</sub> concentration, where the maximum level of silychristin (0.280 mg/g DW) was determined. Enhancement was also detected 6 h (0.142 mg/g DW), 12 h (0.129 mg/g DW) and 48 h (0.099 mg/g DW) after elicitor application. Concentrations c<sub>1a</sub> and c<sub>1b</sub> slightly increased silychristin (0.079 mg/g DW and 0.130 mg/g DW, respectively) after 24 h treatment. The enhancement of other substances of silymarin complex was detected only when the lowest concentration (c<sub>1b</sub>) was applied. The level of silydianin rose in samples harvested 6 h and 12 h after elicitation (0.064 mg/g DW and 0.140 mg/g DW, respectively) and the level of silybin B rose 12 h and 168 h after elicitation (0.131 mg/g DW and 0.110 mg/g DW, respectively).

### Suspensions cultures

Used concentration resulted in increased amounts of taxifolin with maximum 24 h (5.910 mg/g DW) and 48 h (5.189 mg/g DW) after treatment with c<sub>1a</sub> concentration. Elicitor application at c<sub>1a</sub> concentration increased the content of silychristin in samples harvested after 6 h (0.194 mg/g DW) and the content of silydianin after 12 h (0.234 mg/g DW). The maximum values of silychristin and silydianin were monitored 48 h after addition of compound 1 with c<sub>1a</sub> concentration. The c<sub>1a</sub> concentration had no effect on silymarin complex production.

### Table 1: The content of silymarin complex substances (mg/g DW) and taxifolin (mg/g DW) in *Silybum marianum* callus culture after compound 1 elicitation

| Compound 1 (mol/L) | Exposure time (h) | Taxifolin | Silychristin | Silydianin | Silybin A | Silybin B | Isosylbin A | Isosylbin B | Silymarin complex |
|--------------------|------------------|-----------|--------------|------------|-----------|-----------|-------------|-------------|------------------|
| c<sub>1a</sub>=3.292×10⁻³ | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 24 | 0 | 0.079/0.05 | 0 | 0 | 0 | 0 | 0 | 0.079/0.05 |
|                  | 24C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 48 | 0.364/0.05 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 72 | 0.221/0.03 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 168 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 168C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c<sub>1b</sub>=3.292×10⁻⁴ | 6 | 1.419/0.05 | 0.142/0.03 | 0 | 0 | 0 | 0 | 0 | 0.142/0.03 |
|                  | 12 | 0 | 0.129/0.03 | 0 | 0 | 0 | 0 | 0 | 0.129/0.03 |
|                  | 24 | 1.761/0.06 | 0.280/0.04 | 0 | 0 | 0 | 0 | 0 | 0.280/0.04 |
|                  | 24C | 0.064/0.03 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 48 | 0 | 0.099/0.02 | 0 | 0 | 0 | 0 | 0 | 0.099/0.02 |
|                  | 72 | 0.466/0.03 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 168 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 168C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c<sub>1b</sub>=3.292×10⁻⁵ | 6 | 0 | 0 | 0.064/0.03 | 0 | 0 | 0 | 0 | 0.064/0.03 |
|                  | 12 | 0.353/0.05 | 0 | 0.14/0.06 | 0 | 0.131/0.02 | 0 | 0 | 0.271/0.04 |
|                  | 24 | 0.154/0.03 | 0.130/0.03 | 0 | 0 | 0 | 0 | 0 | 0.130/0.03 |
|                  | 24C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 48 | 0.115/0.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 72 | 0.325/0.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|                  | 168 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.110/0.02 |
| 168C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Data are mean/SD. C: Control (without elicitor); 0: Trace amount; SD: Standard deviation; DW: Dry weight.
Flavonolignans in nutrient medium

Flavonolignans were also detected in the nutrient medium in which callus and suspension cultures were cultivated [Table 3]. In case of callus cultures, only silydianin and silybin A at concentration c₁ and c₁a were released into the medium. The lowest concentration of elicitor did not cause flavonolignans releasing. Similar results with a c₁ concentration of elicitor were found in suspension culture medium. Only concentration c₁a and c₁b resulted in the release of taxifolin, silychristin, silydianin, and silybin A into the nutrient medium.

Elicitation with 3-(3-((trifluoromethyl) benzyl) amino) pyrazine-2-carboxamide (2)

Callus culture
Application of all three tested concentrations of compound 2 resulted in an increased production of rutin [Table 4]. The highest production

Table 2: The content of silymarin complex substances (mg/g DW) and taxifolin (mg/g DW) in Silybum marianum suspension culture after compound 1 elicitation

| Compound 1 (mol/L) | Exposure time (h) | Taxifolin | Silychristin | Silydianin | Silybin A | Silybin B | Isosylibin A | Isosylibin B | Silymarin complex |
|-------------------|-------------------|-----------|--------------|------------|-----------|-----------|--------------|--------------|------------------|
| c₁=3.292×10⁻³     | 6                 | 0.334/0.05| 0.194/0.01   | 0          | 0         | 0         | 0            | 0            | 0.194/0.01       |
|                   | 12                | 1.014/0.07| 0            | 0.234/0.03 | 0         | 0         | 0            | 0            | 0.234/0.03       |
|                   | 24                | 0.208/0.03| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 48                | 0.523/0.03| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 72                | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 168               | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 168C              | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |
| c₁a=3.292×10⁻⁴    | 6                 | 0          | 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 12                | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 24                | 5.910/0.08| 0.958/0.06   | 0          | 0         | 0         | 0            | 0            | 0.958/0.06       |
|                   | 48                | 5.189/0.08| 1.618/0.08   | 0.253/0.04 | 0         | 0         | 0            | 0            | 1.871/0.06       |
|                   | 72                | 1.903/0.06| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 168               | 0.588/0.03| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 168C              | 0.049/0.02| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
| c₁b=3.292×10⁻⁵    | 6                 | 0.200/0.03| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 12                | 2.088/0.06| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 24                | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 48                | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 72                | 0.148/0.05| 0            | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 168               | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |
|                   | 168C              | 0          |              | 0          | 0         | 0         | 0            | 0            | 0                |

Data are mean/SD. C: Control (without elicitor), 0: Trace amount, SD: Standard deviation, DW: Dry weight

Table 3: The content of silymarin complex substances (mg/100 mL) and taxifolin (mg/100 mL) in nutrient media of Silybum marianum callus and suspension culture

| Compound 1 (mol/L) | Exposure time (h) | Taxifolin | Silychristin | Silydianin | Silybin A |
|-------------------|-------------------|-----------|--------------|------------|-----------|
|                   | Callus medium     | Suspension medium | Callus medium | Suspension medium |
|                   |                   |             |              |             |           |
| c₁=3.292×10⁻³     | 6                 | 0          |              | 0          | 0         |
|                   | 12                | 0          | 0            | 0          | 0         |
|                   | 24                | 0          | 0            | 0          | 0         |
|                   | 48                | 0          | 0            | 0          | 0         |
|                   | 72                | 0          | 0            | 0          | 0         |
|                   | 168               | 0          | 0            | 0          | 0         |
|                   | 168C              | 0          | 0            | 0          | 0         |
| c₁a=3.292×10⁻⁴    | 6                 | 0          |              | 0          | 0         |
|                   | 12                | 0          | 0            | 0          | 0         |
|                   | 24                | 0          | 0            | 0          | 0         |
|                   | 48                | 0          | 0            | 0          | 0         |
|                   | 72                | 0          | 0            | 0          | 0         |
|                   | 168               | 0          | 0            | 0          | 0         |
|                   | 168C              | 0          | 0            | 0          | 0         |
| c₁b=3.292×10⁻⁵    | 6                 | 0.290/0.04|              | 0          | 0         |
|                   | 12                | 0.240/0.03|              | 1.920/0.07 | 0         |
|                   | 24                | 0          |              | 0          | 0         |
|                   | 48                | 0          |              | 0          | 0         |
|                   | 72                | 0          |              | 0          | 0         |
|                   | 168               | 0          |              | 0          | 0         |
|                   | 168C              | 0          |              | 0          | 0         |

Data are mean/SD. C: Control (without elicitor), 0: Trace amount, SD: Standard deviation, SLB B: Silybin B; ISLB A: Isosylibin A; ISLB: Isosylibin B
was recorded 24 h (0.680 mg/g DW) and 168 h (1.280 mg/g DW) after the addition of the strongest concentration ($c_a$). Higher rutin production was also detected 24, 48, and 72 h after treatment with $c_a$ concentration; 12 h and 24 h after treatment with $c_b$ concentration.

**Suspension culture**

The response of suspension cultures on elicitation was less considerable [Table 4]. The maximum rutin production was detected in samples harvested 168 h after treatment with $c_a$ concentration. Using of $c_a$ concentration slightly increased rutin content after 48 and 72 h (0.040 mg/g DW). The lowest concentration did not significantly affect rutin production.

**Rutin in nutrient medium**

Rutin was only released to the callus nutrient medium after usage of the lowest concentration ($c_a$) [Table 4]. Its maximum value was detected in the case of 24h elicitation (0.670 mg/100 mL). Successful elicitation is subject to many factors that are specific for each elicitor and for each explant at culture. The type and concentration of elicitor, as well as duration time variously, affect metabolite production. Previous works with 5-(2-hydroxybenzoyl)-pyrazine-2-carboxamide[21] and N-(3-iodo-4-methylphenyl)-5-tert-butyl-pyrazine-2-carboxamide[22] have shown to be an effective elicitors of flavonolignans, predominantly silychristin and taxifolin, in *S. marianum* callus and suspension cultures. Other pyrazine derivatives increased the level of flavonoid in *O. arvensis* or level of isoflavonoid in *Trifolium pratense* in vitro cultures.[23]

### Table 4: The content of rutin (mg/g DW) in *Fagopyrum esculentum* callus and suspension culture and the content of rutin (mg/100 mL) in callus nutrient medium after compound 2 elicitation

| Compound (mol/L) | Exposure time (h) | Callus culture | Suspension culture | Callus medium |
|------------------|------------------|----------------|-------------------|---------------|
| $c_a = 3.3756 \times 10^{-3}$ | 6 | 0 | 0.030/0.01 | 0 |
|                  | 12 | 0.320/0.02 | 0 | 0 |
|                  | 24 | 0.680/0.04 | 0.030/0.01 | 0 |
|                  | 48 | 0.010/0.01 | 0.020/0.01 | 0 |
|                  | 72 | 0.070/0.02 | 0.040/0.02 | 0 |
|                  | 168 | 1.280/0.06 | 0 | 0 |
|                  | 168C | 0.010/0.01 | 0 | 0 |
| $c_b = 3.3756 \times 10^{-4}$ | 6 | 0 | 0.010/0.01 | 0 |
|                  | 12 | 0.010/0.02 | 0 | 0 |
|                  | 24 | 0.350/0.06 | 0.010/0.01 | 0 |
|                  | 48 | 0.480/0.04 | 0 | 0 |
|                  | 72 | 0.490/0.04 | 0 | 0 |
|                  | 168 | 0.020/0.01 | 0.320/0.02 | 0 |
|                  | 168C | 0.060/0.02 | 0.090/0.05 | 0 |
| $c_{b2} = 3.3756 \times 10^{-5}$ | 6 | 0.40/0.05 | 0.010/0.01 | 0.140/0.06 |
|                  | 12 | 0.260/0.02 | 0 | 0.060/0.04 |
|                  | 24 | 0.120/0.03 | 0.010/0.01 | 0.670/0.04 |
|                  | 48 | 0 | 0 | 0.020/0.01 |
|                  | 72 | 0.0010/0.01 | 0 | 0.330/0.02 |
|                  | 168 | 0 | 0 | 0.060/0.03 |
|                  | 168C | 0.010/0.01 | 0.010/0.02 | 0.050/0.03 |

Data are mean/SD. C: Control (without elicitor), O: Trace amount; SD: Standard deviation; DW: Dry weight.

### CONCLUSION

Elicitation with compound 1 on *S. marianum* cultures resulted in enhanced flavonolignan productions, taxifolin and silychristin, mainly when $c_a$ ($3.292 \times 10^{-4}$ mol/L) concentration of elicitor was used. From the results of the performed experiments, it can be concluded that compound 2 shows to be a suitable elicitor for increasing rutin production in callus cultures but less appropriate for suspension cultures of *F. esculentum*.

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### Conflicts of interest

There are no conflicts of interest.

### REFERENCES

1. Khan V, Walterova D. Silybin and silymarin – New effects and applications. Biomed Pap 2005;149:29-41.
2. Shaker E, Mahmoud H, Mhas S. Silymarin, the antioxidant component and *Silybum marianum* extracts prevent liver damage. Food Chem Toxicol 2010;48:803-6.
3. Vaid M, Singh T, Pasad R, Katiyar SK. Silymarin inhibits melanoma cell growth both in vitro and in vivo by targeting cell cycle regulators, angiogenic biomarkers and induction of apoptosis. Mol Carcinog 2014: doi: 10.1002/mc.22208. [Epub ahead of print].
4. Perez HJ, Carrillo SC, Garcia E, Ruiz-Marin G, Perez-Tamayo R, Chavarria A. Neuroprotective effect of silymarin in a MPTP mouse model of Parkinson’s disease. Toxicology 2014;319:38-43.
5. Singhal NK, Srivastava G, Patel DK, Jain SK, Singh MP. Melatonin or silymarin reduces manganese and paraquat-induced Parkinson’s disease phenotype in the mouse. J Pineal Res 2011;50:97-109.
6. Inglee GE, Chen D, Berhow M, Lee S. Anti-oxidant activity of commercial buckwheat flours and their free and bound phenolic compositions. Food Chem 2011;125:923-9.
7. Vogrinicic M, Kreft I, Filipic M, Zegura B. Antigenotoxic effect of Tartary (*Fagopyrum tataricum*) and common (*Fagopyrum esculentum*) buckwheat flour. J Med Food 2013;16:944-52.
8. Orlic D, Svitieva E, Mimica-Dukic N, Beare I, Balog K, Franciković M, et al. Phenolic profile and anti-oxidant activity of buckwheat (*Fagopyrum esculentum*) herb and root extracts. Planta Med 2012;78:11.
9. Namdeo AG. Plant cell elicitation for production of secondary metabolites: A review. Pharmacogn Rev 2007;1:69-79.
10. Zhao J, Davis LC, Verpoorte R. Elicitor signal transmission leading to production of plant secondary metabolites. Biotechnol Adv 2005;23:283-333.
11. Sánchez-Sampedro MA, Fernández-Tarrago J, Corchete P. Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn. J Biotechnol 2005;119:60-9.
12. Zhao G, Zhao J, Peng L, Zou L, Wang J, Zhong L, et al. Effects of yeast polysaccharide on growth and flavonoid accumulation in *Fagopyrum tataricum* sprout cultures. Molecules 2012;17:1135-45.
13. Elwekee A, Elfishawy A, AbouZid S. Enhanced accumulation of flavonolignans in *Silybum marianum* cultured roots by methyl jasmonate. Phytother Res 2010;5:390-6.
14. Kim HJ, Park KJ, Lim JH. Metabolicomic analysis of phenolic compounds in buckwheat (*Fagopyrum esculentum*) sprouts treated with methyl jasmonate. J Agric Food Chem 2011;59:5707-13.
15. Khalili M, Hasnando T, Kazem Tabar SK, Rahama H. Influence of exogenous salicylic acid on flavonolignans and lipoygenase activity in the hairy root cultures of *Silybum marianum*. Cell Biol Int 2009;33:988-94.
16. Ashtiani SR, Hasnando T, Bhamta MR. Enhanced production of silymarin by Ag+ elicitor in cell suspension cultures of *Silybum marianum*. Pharm Biol 2010;48:708-15.
M. tuberculosis in vitro in monocytes and in mice by aminomethylene pyrazinamide analogs. Tuberculosis (Edinb) 2008;88:410-9.

19. Servusová B, Eibinová D, Doležal M, Kubicek V, Paterová P, Pesko M, et al. Substituted N-benzylpyrazine-2-carboxamides: Synthesis and biological evaluation. Molecules 2012;17:13183-96.

20. Doležal M, Kráľová K. Herbicides, Theory and Applications: Synthesis and Evaluation of Pyrazine Derivatives with Herbicidal Activity. InTech; 2011. Available from: http://www.intechopen.com/books/herbicides‑theory‑and‑applications/synthesis‑andevaluation‑of‑pyrazine‑derivatives‑with‑herbicidal‑activity.

21. Tumová L, Tuma J, Megusar K, Doležal M. Substituted pyrazinecarboxamides as abiotic elicitors of flavolignan production in Silybum marianum (L.) gaertn cultures in vitro. Molecules 2010;15:33140.

22. Tumova L, Tuma J, Dolezal M. Pyrazinecarboxamides as potential elicitors of flavonolignan and flavonoid production in Silybum marianum and Ononis arvensis cultures in vitro. Molecules 2011;16:9142-52.

23. Doležal M, Zitko J, Canillo C, Eibinová D, Tauchman M, Kuneš J, et al. Substituted N-Benzylpyrazine-2-Carboxamides, their Synthesis, Hydro-Lipophilic Properties and Evaluation of their Anti-Mycobacterial, and Photosynthesis-Inhibiting Activity. 15th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-15). November 1-30, 2011. p. a64/1-a64/6.

24. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:437-97.

25. The Ministry of Health of the Czech Republic. Fagopyrherba. In: Czech Pharmacopoeia. Praha: Grada; 2009. p. 2069-70.

26. Dolezal M, Tumová L, Kšetovcová D, Tuma J, Králová K. Substituted N-phenylpyrazine-2-carboxamides, their synthesis and evaluation as herbicides and abiotic elicitors. Molecules 2007;12:2589-98.

27. Kalparová M, Siatak T, Klimešová V, Dušek J. New synthetic pyridine derivate as potential elicitor in production of isoflavonoids and flavonoids in Trifolium pratense L. suspension culture. ScientificWorldJournal 2012;2012:346412.

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