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ORIGINAL ARTICLE

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

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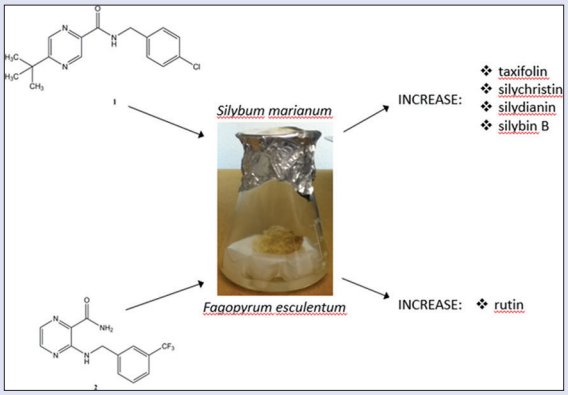
ABSTRACT

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 flavonolignan and flavonoid production in *S. marianum* and *Fagopyrumes culentum in vitro* cultures. **Materials and Methods:** Callus and suspension cultures were cultured on MS medium containing α-naphtaleneacetic 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-carboxamidesderivatives on secondary metabolite content of *Silybum marianum* and *Fagopyrum esculentum* in vitro cultures was tested.
- In *S. marianum*, the derivate N-(4-chlorobenzyl)-5-tert-butylpyrazine-2-carboxamide showed as a good elicitor of taxifolin production and less effective for silymarin complex production with silychristin as the most abundant.
- The derivate 3-(3-((trifluoromethyl) benzyl) amino) pyrazine-2-carboxamide is suitable for increase rutin production in callus cultures and less appropriate for suspension cultures of *F. esculentum*.



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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.[1] 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,[2,3] neuroactive, and neuroprotective.[4] Its anti-oxidant activity is due to its free radical scavenging property, an increase in superoxide dismutase activity[5] and the inhibition of lipid peroxide formation.[6]

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 benefic al for human health.[7,8] 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.[9]

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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 modifi ation 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.[10,11] 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 signifi ance. 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 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 Kralove 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 fi ld 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 F_{254} TLC plates (Merck, Darmstadt, Germany). Compounds were purifi d using an automated chromatograph CombiFlashR_f (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 ^{1}H and 75 MHz for ^{13}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 imited, Staffordshire, UK) and are uncorrected.

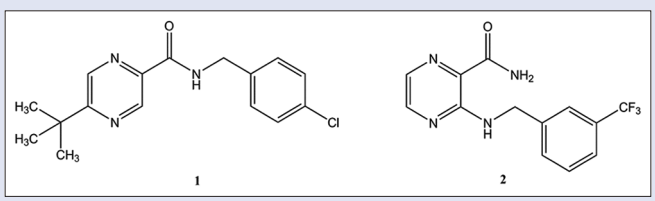

Figure 1: Structures of pyrazine elicitors 1 and 2

Synthesis of pyrazine elicitors 1 and 2

N-(4-chlorobenzyl)-5-tert-butylpyrazine-2-carboxamide (1) was synthesized through aminolysis of 5-tert-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-tert-butylpyrazine-2-carboxamide (1). White crystalline compound. Yield: 83%; m.p. 95.1–96.5°C; ^{1}H-NMR ($CDCl_3$) δ 9.32 (s, 1H, H3), 8.54 (s, 1H, H6), 8.10 (bs, 1H, NH), 7.34–7.25 (4H, m, ArH), 4.63 (2H, d, J = 6.2 Hz, CH_2), 1.41 (9H, t, J = 0.6 Hz, *tert*-butyl H); ^{13}C-NMR ($CDCl_3$) 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 $C_{16}H_{18}ClN_3O$ (303.79): 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; ^{1}H-NMR ($CDCl_3$) δ 9.23 (1H, t, J = 6.1 Hz, NH), 8.22 (1H, d, J = 2.2 Hz, H6), 8.19 (1H, bs, NH_2), 7.80 (1H, d, J = 2.2 Hz, H5), 7.72 (1H, bs, NH_2), 7.66 (1H, s, H2′), 7.64–7.50 (3H, m, H4′, H5′, H6′), 4.74 (2H, d, J = 6.1 Hz, CH_2); ^{13}C-NMR ($CDCl_3$) 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 = 272.0 Hz), 123.8 (q, J = 3.9 Hz), 123.7 (q, J = 6.1 Hz), 43.1; IR (cm^{-1}) 3468 (NH_2), 1674 (C = O); Anal. Calcd. for $C_{13}H_{11}F_3N_4O$ (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 germinating 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 MS[24] medium containing α-naphtaleneacetic 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^{-3} 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-tert-butyl-pyrazine-2-carboxamide (compound 1) at concentrations 3.292×10^{-3} mol/L (c_1); 3.292×10^{-4} mol/L (c_{1a}); 3.292×10^{-5} mol/L (c_{1b}); and 3-(3-((trifluoromethyl) benzyl) amino) pyrazine-2-carboxamide (compound 2) at concentrations 3.3756×10^{-3} mol/L (c_2); 3.3756×10^{-4} mol/L (c_{2a}); 3.3756×10^{-5} mol/L (c_{2b}) were used. Elicitors were dissolved in 96% ethanol, and 1 mL of solution was

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 (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 0.02 μm microfilter, and approximately 1.7 mL of filtrate was analyzed by HPLC with DAD detection. The HPLC method was modifi d at the department according to the Czech Pharmacopoeia.[25]

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 $P \leq 0.05$ were considered as signifi antly 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_{1a}. The lowest production of taxifolin was monitored, when the highest concentration of elicitor was used. The best elicitation effect in callus culture was found 24 h after treatment with c_{1a} 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_1 and c_{1b} 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_{1b}) 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_{1a} concentration. Elicitor application at c_1 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_{1a} concentration. The c_{1b} 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	Isosylibin A	Isosylibin B	Silymarin complex
$c_1=3.292\times10^{-3}$	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
$c_{1a}=3.292\times10^{-4}$	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	0.061/0.04	0	0	0	0	0	0	0
$c_{1b}=3.292\times10^{-5}$	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	0.1150.02	0	0	0	0	0	0	0
	72	0.325/.02	0	0	0	0	0	0	0
	168	0	0	0	0	0.110/0.02	0	0	0.110/0.02
	168C	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 $c_1$and c_{1a} were released into the medium. The lowest concentration of elicitor did not cause flavonolignans releasing. Similar results with a c_1 concentration of elicitor were found in suspension culture medium. Only concentration c_{1a} and c_{1b} resulted in the release of taxifolin, silychristin, silydianin, and sylibin 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
$c_1=3.292\times10^{-3}$	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
	24C	0	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	0
	168	0	0	0	0	0	0	0	0
	168C	0	0	0	0	0	0	0	0
$c_{1a}=3.292\times10^{-4}$	6	0	0	0	0	0	0	0	0
	12	0	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
	24C	0.050/0.02	0	0	0	0	0	0	0
	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
$c_{1b}=3.292\times10^{-5}$	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	0
	24C	0	0	0	0	0	0	0	0
	48	0	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	0
	168C	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

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	Callus medium	Suspension medium	Callus medium	Suspension medium
$c_1=3.292\times10^{-3}$	6	0	0	0	0	0	0	0	0
	12	0	0	0	0	0.220/0.03	0	0	0
	24	0	0	0	0	0	0	0.380/0.03	0
	24C	0	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0	0
	72	0	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
$c_{1a}=3.292\times10^{-4}$	6	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0
	24C	0	0	0	0	0	0	0	0
	48	0	0.380/0.03	0	0	0	0	0	0
	72	0	0	0	0	0	0	0	0
	168	0	0.240/0.03	0	0	0.790/0.05	0.790/0.04	0	0
	168C	0	0	0	0	0	0	0	0
$c_{1b}=3.292\times10^{-5}$	6	0	0.290/0.04	0		0	0	0	0
	12	0	0.240/0.03	0	1.920/0.07	0	0.710/0.04	0	0.190/0.03
	24	0	0	0		0	0	0	0
	24C	0	0	0	0.290/0.03	0	0	0	0
	48	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	0.280/0.03	0	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

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 2 (mol/L)	Exposure time (h)	Rutin (mg/g DW)		Rutin (mg/100 mL)
		Callus culture	Suspension culture	Callus medium
$c_2=3.3756\times10^{-3}$	6	0	0.030/0.01	0
	12	0.320/0.02	0	0
	24	0.680/0.04	0.030/0.01	0
	24C	0.010/0.01	0.020/0.01	0
	48	0.010/0.01	0.040/0.02	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
$c_{2a}=3.3756\times10^{-4}$	6	0.010/0.01	0.010/0.01	0
	12	0.010/0.02	0	0
	24	0.350/0.06	0.010/0.01	0
	24C	0	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
$c_{2b}=3.3756\times10^{-5}$	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
	24C	0	0	0.020/0.01
	48	0.010/0.01	0	0.340/0.02
	72	0.010/0.01	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), 0: Trace amount; SD: Standard deviation; DW: Dry weight

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_2). Higher rutin production was also detected 24, 48, and 72 h after treatment with c_{2a} concentration; 12 h and 24 h after treatment with c_{2b} 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_{2a} concentration. Using of c_2 concentration slightly increased rutin content after 48 and 72 h (0.040 mg/g DW). The lowest concentration did not signifi antly affect rutin production.

Rutin in nutrient medium

Rutin was only released to the callus nutrient medium after usage of the lowest concentration (c_{2b}) [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[20] 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*[22,26] or level of isoflavonoid in *Trifolium pratense in vitro* cultures.[27]

CONCLUSION

Elicitation with compound 1 on *S. marianum* cultures resulted in enhanced flavonolignan productions, taxifolin and silychristin, mainly when c_{1a} (3.292×10^{-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 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 confli ts of interest.

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