The effect of substituted amides of pyrazine-2-carboxylic acids on flavonolignan production in *Silybum marianum* culture *in vitro*

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**Abstract**

*In vitro* plant tissue and cell cultures were used to study herbicide effects on growth, selected metabolic activities and other phenomena. The effect of abiotic elicitors, two newly synthesized substituted amides of pyrazine-2-carboxylic acids (synthesized at the Department of Pharmaceutical Chemistry and Drug Control, School of Pharmacy in Hradec Kralove), on the flavonolignan accumulation in callus and suspension culture of *Silybum marianum* (L.) Gaertn. was investigated. The compounds markedly influenced production of flavonolignans in an *in vitro* culture. Particularly after the elicitation with a solution of compound 3-methylamide 5-tert-butylpyrazine-2-carboxylic acid at a concentration of 3.71x10⁻⁷ mol l⁻¹ and within 72 h of elicitation, an increase in flavonolignan production by 893 % in suspension culture versus control took place. The flavonolignan accumulation in callus culture after the elicitation with a solution of 5-brom-2-hydroxyphenylamide of 5-tert-butyl-6-chloropyrazine-2-carboxylic acid was also increased by about 1039 % (24 hour elicitation and concentration of 2.59x10⁻⁴ mol l⁻¹).

**List of abbreviations:**

DNOC 2-methyl-4,6-dinitrophenol

2,4-D - 2,4-dichlorophenoxyacetic acid

HPLC - high-performance liquid chromatography

NAA - α-naphthaleneacetic acid

**Introduction**

Plant tissue and cell cultures provide model systems for the study of various molecular, physiological, systemic and genetic problems. These systems were used in studies with herbicides and other xenobiotics (Dwight Camber and McDonald 1989).

Herbicides are generally considered to be inhibitors, thus different inhibitory responses have been studied in various culture systems.

Callus tissues, particularly of tobacco, have been studied to test various compounds for cytokine activity (Linsmaier and Skoog 1965, Schmitz *et al.* 1972). It presents a convenient bioassay for substances that inhibit cell division because differentiation is minimized and cell division is the primary morphological event. In a study of the response of tobacco callus to 11 different dinitroanilines,
dinitramine produced the highest inhibitory effect on growth (fresh and dry mass) (Huffman and Camber 1978).

A study of Blein (1982) evaluated the activity of 15 herbicides from different chemical families in non photosynthetic sycamore cells. Clorthiamid, propanil, flurodifen, nitrophen, dichlorobenil, ioxynil, dinoseb and DNOC had lethal effects. Ametryn, atrazine, desmetryne, bromacil, lenacil and terbacil inhibited growth to some extent, whereas metribuzin was stimulatory. Some compounds strongly stimulated oxygen consumption (ioxynil, dinoseb and DNOC); propanil, flurodifen, dichlofenil and atrazine had lesser effects. The other herbicides listed above had no effect.

Ring substituted pyrazine-2-carboxamides were tested for their in vitro antimycobacterial, antifungal and photosynthesis-inhibiting activities. The 3-methylphenylamides of 6-chloro- and 5-tert-butyl-6-chloropyrazine-2-carboxylic acid exhibited only a poor in vitro antifungal effect against all tested strains (Candida albicans ATCC 44859, Candida tropicalis 156, Candida Krusei E 28, Candida glabrata 20/I, Trichosporon beigelii 1188, Trichophyton mentagrophytes 445, Aspergillus fumigatus 231 and Absidia corymbifera 272), although the latter was the most active antialgal compound. The most active inhibitor of oxygen evolution rate in spinach chloroplasts in this series was the 3,5-bis(trifluoromethylphenyl)amide of 6-chloropyrazine-2-carboxylic acid (Dolezal et al. 2002). The introduction of chlorine in the pyrazine moiety led to an increased photosynthesis-inhibiting activity (Dolezal et al. 1999). A series of pyrazine carboxylic acid derivatives was tested in order to verify the role of the diazine ring and its substituents on auxinic activity. This activity was examined using three tests: pea stem elongation, flax root inhibition and ethylene production in etiolated pea stem. All tested compounds were practically inactive, except the 3-amino-6-chloropyrazine-2-carboxylic acid, which showed anti-auxin behaviour (Ricci et al. 1991). All substituted amides of pyrazine-2-carboxylic acid can be interpreted as some aza-analogues of nicotinamide. The effect of abiotic elicitors, i.e. two newly synthesized compounds (at the Department of Pharmaceutical Chemistry and Drug Control, School of Pharmacy in Hradec Kralove, Czech Republic), both of them substituted amides of pyrazine-2-carboxylic acids, on the formation of flavonoids was also tested in the callus culture of Ononis arvensis L.. These compounds markedly influenced the production of flavonoids in an in vitro culture. Particularly after elicitation with a solution of compound 4-hydroxamide of 6-chloro-5-tert-butylpyrazine-2-carboxylic acid at a concentration of 3.32 x 10^{-7} mol l^{-1} and within 48 h of elicitation, an increase in flavonoid content by 976 % versus the control took place (Tumova and Ostrozlik 2002).

Silymarin, derived from Silybum marianum (L.) Gaertn., is a complex group of flavonolignans—addition compounds of flavononols and coniferyl alcohol. The principal components are silybin, isosilybin, silychristin, silydianin and their derivatives (Wichtl 1997). Silymarin has been used for centuries as a natural remedy for diseases of the liver and biliary tract. Silymarin and its active constituent, silybin, have been reported to work as antioxidants scavenging free radicals and inhibiting lipid peroxidation. Studies also suggest that they protect against genomic injury, increase hepatocyte protein synthesis, decrease the activity of tumor promoters, stabilize mast cells, chelate iron, and slow calcium metabolism (Flora et al. 1998).

In this study, the influence of two substituted amides of pyrazine-2-carboxylic acids on the production of silymarin in Silybum marianum (L.) Gaertn. culture in vitro was investigated.

**Materials and methods**

**Biological material**

Callus culture was derived from the germinating seeds of plant Silybum marianum (L.) Gaertn. (Asteraceae). Seeds for germination were obtained from the Garden of Medicinal Plants, School of Pharmacy in Hradec Kralove. One-week-old seedlings were used for inoculation of plant material to Murashige-Skoog medium (MS) (Murashige and Skoog 1962). Suspension culture was established from 3-month-old undifferentiated hypocotyl calluses.