

THE EFFECT OF COPPER SULPHATE ON THE PRODUCTION OF FLAVONOIDS IN *DIGITALIS LANATA* CELL CULTURES

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Abstract

The paper investigated the effect of abiotic elicitor copper sulphate on the production of flavonoids in cell cultures of *Digitalis lanata*. Two cell lines were used (line 11 and line 13C-100) and two types of experiments were performed. In the first type of experiment the highest production of flavonoids was established, for both cell lines, for the strongest elicitor concentration (8 μ M). In the second type of experiment the maximal flavonoid production was induced for line 11 after 24 hour elicitation (over 10 times more compared with the control, from 0.624mg/g dry weight (d.w.) to 6mg/g d.w.), for the highest elicitor concentration (40 μ M).

Rezumat

În lucrarea de față am testat efectul sulfatului de cupru, un elicitor abiotic asupra producției de flavone în culturi celulare de *Digitalis lanata*. Au fost efectuate două tipuri de experimente, folosind două linii celulare de *Digitalis lanata*: linia 11 și linia 13C-100. Pentru primul tip de experiment, cele mai mari cantități de flavone se acumulează, pentru ambele linii celulare, la concentrația cea mai mare a elicitorului (8 μ M).

În ceea ce privește al doilea tip de experiment, linia 11 sintetizează cantitatea cea mai mare de flavone (de aproximativ 10 ori mai mult comparativ cu martorul, de la 0,624mg/g s.u. (substanță uscată) la 6mg/g s.u., după 24 ore de elicitare, folosind concentrația cea mai mare de sulfat de cupru, 40 μ M).

Keywords: abiotic elicitors, flavonoids production

Introduction

Plants express a number of morphological and physiological responses to a range of physical and chemical factors from the environment, factors known as elicitors. These responses are considered as defense reactions elaborated by plants enzymatic systems to ensure their survival, persistence and competitiveness [11].

Secondary metabolism compounds synthesised as defense molecules are called phytoalexins. Most of these substances have low molecular weight and many of them are flavonoids [6].

Plants cultivated *in vitro*: calli cultures, tissue cultures and cell cultures (as suspensions) have the same behaviour as plants to elicitors.

Phytoalexins are absent or present in low amounts in healthy plants

or *in vitro* plants cultures[1]. In the presence of elicitors the amounts of phytoalexins can increase very much (up to ten times).

The salts of many heavy metals (Ag, V, Cr, Ni, Pb, Hg) or recently, salts of Al and Cd were tested as elicitors [8,12,15-18]. Among them copper salts (as sulphate or chloride) were successfully used as abiotic elicitors in a number of plant cell cultures[7,9,14].

Previously, we investigated the effect of copper sulphate on the accumulation of cardiac glycosides in cell cultures of *Digitalis lanata*. The results were very promising (the level increased up to 26 times when compared with the control)[2]. In this paper we investigated the effect of copper sulphate on the production of flavonoids in *Digitalis lanata* cell cultures.

Materials and methods

Plant material

The experiments were performed on two cell lines of *Digitalis lanata*: line 11 and line 13C-100, obtained as reported previously [3].

Suspension cultures have been maintained on Murashige-Skoog medium [10] with 1mg/L 2,4-dichlorophenoxyacetic acid and 2mg/L benzylaminopurine in 300mL Erlenmeyer flasks containing 50mL medium. They were subcultivated every 14 days, the ratio *innoculum* to fresh medium was 1:6. The flasks were kept on a gyratory shaker at 100 rpm to a photoperiod of 16 hours, with illumination of 35 μ mol photons m⁻²s⁻¹ and a temperature of 25°C.

Flavonoids analysis was performed in accordance with the Romanian Pharmacopoeia, by a spectrophotometric method (*Cynarae folium* monography) using aluminium chloride as reagent and a calibration curve developed with rutoside.[4,5,11,13].

We performed two types of experiments. The first type of experiment: 1 mL CuSO₄ was added in flasks containing 0 days suspension cultures to obtain a final copper concentration of 2, 4 and 8 μ M respectively. The cells were harvested after 12 days. The second type of experiment: CuSO₄ was added in flasks containing 10 days suspension cultures to obtain a final copper concentration of 20 and 40 μ M respectively. The cells were harvested after 24, 48 and 72 hours respectively.

Results and discussion

The succes of elicitation depends upon a number of factors, each of them specific for a certain elicitor and a certain type of plant cell culture such as: elicitor concentration, elicitation time, the age of suspension culture when adding the elicitor.

Based on previous experiments we performed two types of experiments and tested some copper sulphate concentrations (as shown previously), using two different ages of plant cell cultures (0 and 10 days suspension cultures) and the elicitor action time also varied (as shown previously).

For the first type of experiment we recorded increases of flavonoids production for all elicitor concentrations and for both cell lines. The flavonoids amount grew with the copper salt concentration. Thus, the comparison of the flavonoids production in the elicited *Digitalis lanata* suspension cultures (lines 11 and 13C-100) showed that the maximum increase in the production of flavonoids in both cases was induced after the application of the highest concentration of copper sulphate, 8 μ M respectively. The highest content of flavonoids was recorded for line 11, over 9 times more compared with the control from 0.624mg/g dry weight(d.w.) to 6mg/g d.w. For line 13C-100 the growth was of approximately 2.5 times more, when compared with control, from 2.86mg/g d.w. to 7.65mg/g d.w. (fig.1).

These results are in accordance with other results reported using copper salts as elicitors. Thus, in *Trifolium pratense* cell cultures it was observed that the best elicitation effect was achieved after 10 days of elicitation with the highest elicitor concentration (10 μ M CuSO₄) [7]. Also, these results are in accordance with the results we obtained for cardiac glycosides accumulation in *Digitalis lanata* cell cultures eliciting with copper sulphate [2]. In that experiment line 11 accumulated the highest digitoxin amount for the highest CuSO₄ concentration, 40 μ M (over 20 times more compared with the control, from 0.6mg/g d.w. to 12.3mg/g d.w.).

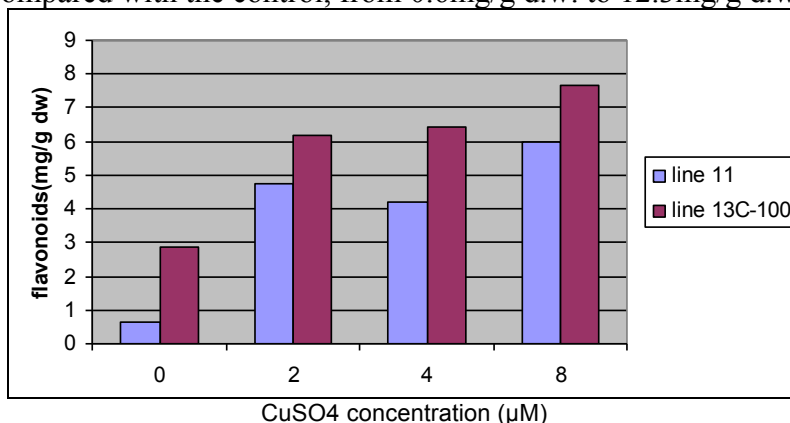


Figure 1

The variation of flavonoids synthesis within the two cell lines used. First experiment.

The second type of experiment: For line 11, the best elicitation effect was recorded for the highest copper concentration (40 μ M) for all tested time intervals (24, 48 and 72 hours respectively). For the highest concentration, line 11 accumulated the greatest flavonoids content after 24 hours elicitation (almost 10 times more compared with the control, from 0.43mg/g d.w. to 4.15mg/g d.w.).

The literature reports similar result. In *Ononis arvensis* suspension cultures, the flavonoids production was significantly increased after 24, 48 and 72 hours elicitation with CrCl₃ in the highest concentration (6.32x10⁻⁴ mol/L) [15].

For the lowest CuSO₄ concentration, 20 μ M, line 11 accumulated the highest amount of flavonoids after 72 hours of elicitation, from 0.62mg/g d.w. to 3.43mg/g d.w. (fig.2).

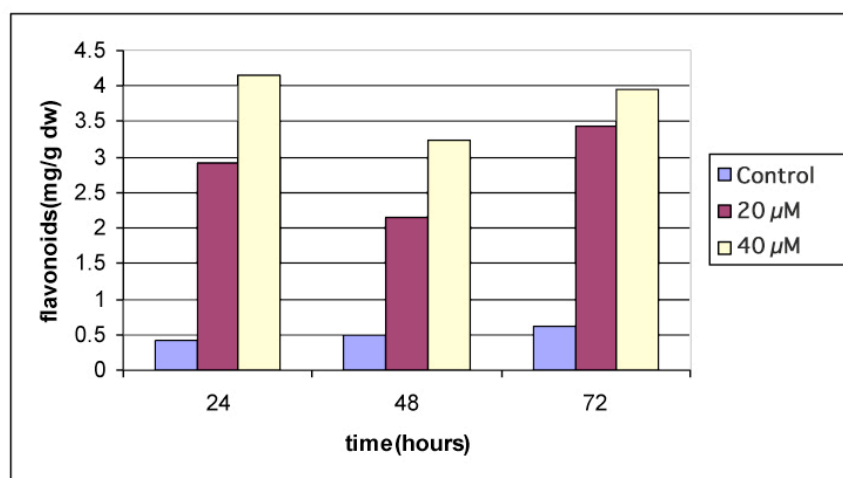


Figure 2

The variation of flavonoids synthesis in line 11, in the second experiment.

Similar reports were found in literature. In hairy roots cultures of *Panax ginseng*, the addition of 20 μ M of NiSO₄ resulted in an increase in ginseng saponin content to about 1.2 times compared with the control levels [6].

For line 13C-100 the maximum increase in the flavonoids accumulation was induced after 24 hours application of the highest CuSO₄ concentration 40 μ M, from 2mg/g d.w. to 4.3 mg/g d.w. After 24 hours, the flavonoids content decreased for both elicitor concentrations, 20 and 40 μ M respectively (fig. 3).

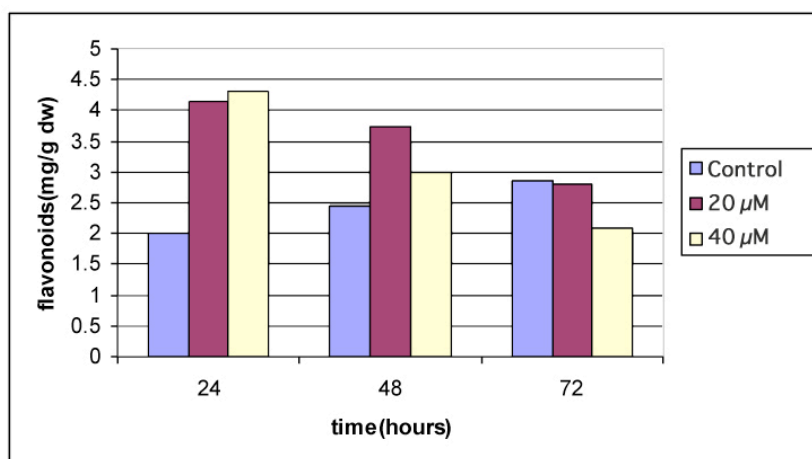


Figure 3

The variation of flavonoids synthesis line 13C-100, in the second experiment

Similary, the literature reports in *Ononis arvensis* suspension cultures elicited with nichel and cobalt salts, flavonoid production increased after 24 hours elicitation (similar results were obtained in callus cultures of *Ononis arvensis* eliciting with AgNO_3) [17]. Comparing the two cell lines in the second type of experiment, the best responses were obtained for the highest copper sulphate concentration, 40µM. Line 11 response was higher than line 13 C-100 response at the CuSO_4 action as elicitor, so line 11 suspension cultures accumulated the highest flavonoids amount, 4.15mg/g d.w. after 24 hours elicitation with 40 µM CuSO_4 (fig. 2 and fig. 3).

Conclusions

The results obtained prove that copper sulphate is suitable to be used as elicitor in *Digitalis lanata* suspension cultures, the same as in other plants suspension cultures, to stimulate the secondary metabolites production.

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