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MYCOTOXIN AND DROUGHT STRESS INDUCED CHANGE OF ALKALOID CONTENT OF *PAPAVER SOMNIFERUM* PLANTLETS

B. SZABÓ[1], E. TYIHÁK[2], L. GY. SZABÓ[1] and L. BOTZ[3]

[1]*Department of Botany, University of Pécs*
H-7624 Pécs, Ifjúság u. 6, Hungary, E-mail: csele@pointer.hu
[2]*Plant Protection Institute, Hungarian Academy of Sciences*
H-1022 Budapest, Herman O. u. 15, Hungary
[3]*Department of Pharmacy, Faculty of General Medicine, University of Pécs*
H-7624 Pécs, Honvéd u. 3, Hungary

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Papaver somniferum produces secondary metabolites, which have important roles in their self-defence processes, in plant biochemistry and in allelochemistry. We can see that different stress effects change the quantity of alkaloids. The object of the experiments is, in what manner changes the content of alkaloids of poppy in case of irregular stress effects. *Papaver somniferum* (cv. 'Kék Duna', Budakalász) plants were grown for 2 months from seeds in quartz-sand (in natural light, temperature: 24–28 °C, in Knop's nutritive solution). In this paper we studied the alkaloid of poppy treated with two kind of stress factors: mycotoxin and drought, respectively. Both the quantity and the spectrum of alkaloids were measured after different separation procedures. Thin layer chromatography (TLC and HPTLC) and high performance liquid chromatography (HPLC) were applied. Content of the level of formaldehyde (HCHO) also increases in plants with different stress effects. Our presupposition is that the formation of methyl groups of poppy alkaloids takes place through HCHO. It gave us an opportunity to examine changing of formaldehyde (HCHO) level in biotic and abiotic stress situation. Formaldehyde in dimedone adduct form can be detected in injured tissues of *Papaver somniferum*. As a consequence, the stress effects can be detected in poppy plants by two kinds of method. At first we measured content of alkaloids. Drought stress produced a higher level of the alkaloids, but the mycotoxin stress did not show significant results.

Key words: formaldehyde, HPLC, morphine alkaloids, stress effects, TLC

INTRODUCTION

Plants produce secondary metabolites that have important roles in their self-defence processes, in plant biochemistry and in allelochemistry (Reigoza *et al.* 1999). Allelopathy, the chemical mechanism of plant interference, is characterised by a reduction in plant emergence or growth, resulting in a reduced performance of the plant in question. For better understanding of allelopathy extensive research has been done regarding the environmental and stress ef-

fects. The purpose of these experiments was to decide which factors could have major influence on the production of secondary metabolites.

Most of the natural products that cause allelopathy are a subset of the array of secondary compounds synthesised by plants and microorganisms, and most of the currently identified compounds are products of the shikimic acid and acetate pathway (Rice 1984). The common ones include phenolics like cinnamic and benzoic acids, coumarins, tannins, and flavonoids; terpenoids; and a few alkaloids, steroids, and quinones (Einhelling and Leather 1988). Although most of the simple phenolic acids and flavonoids are known to be allelochemicals, they seem to be weekly phytotoxic in soil and have little selectivity. Many of the phenolic compounds at very high rates are effective against weeds and are relatively non-selective (Duke and Lyndon 1993). However, the authors suggest that synthetic modification of these compounds increase their efficacy and selectivity. In this respect, the halogenated benzoic acid herbicides like dicamba, chloramben, and picloram are derived from benzoic acid, a phenolic plant product. Naphtoquinones such as juglone and lawsone are among the more phytotoxic phenolic compounds (Spencer *et al.* 1986). Phenolic derivatives, such as the dihydroquinone sorgoleone, produced by *Sorghum bicolor*, are extremely phytotoxic in hydroponic culture (Einhelling and Souza 1992). The importance of allelopathy in nature and in agroecosystem has attracted researcher's attention with the main goal of using the phenomenon in biological control of weeds. Currently, active involvement of scientists from different disciplines made allelopathy a multidisciplinary subject, and transformed the research from basic to applied, enabling use of allelopathy in agriculture and forestry. Screening accession of allelopathic crops and natural vegetation for their ability to reduce weeds is the basic approach for utilising the phenomenon. For example, coffee (*Coffea arabica* L.) and tea (*Camellia sinensis* (L.) O. Kuntze) are known for toxic alkaloid production and their possible allelopathic or autotoxic influences (Rizvi *et al.* 1981, Waller *et al.* 1986, Rizvi and Rivzi 1987, Suzuki and Waller 1987). High concentration of 1, 3, 7-trimethylxanthine (1,3,7-T) or caffeine has been isolated from soil around coffee trees. They also found related purine alkaloids and fatty acids, which demonstrated allelopathic activity. Rizvi *et al.* (1981) proposed that since caffeine exerted differential action on several plant species, it might be a useful selective herbicide. Rizvi and Rizvi (1992) also found reduction in amylase activity in germinating seeds of *Amaranthus spinosa* after treating with 1,3,7-T. They also found that the alkaloid inhibited seven other noxious weeds at various concentration.

Papaver somniferum contains about known 50 alkaloids. The main alkaloids of them are morphine, codeine, papaverine, tebaine and narkotine. There-

fore, it seemed promising to treat poppy plantlets with different stress factors and to monitor plantlet response by examination of their alkaloid contents.

In this paper we studied the alkaloids of poppy treated with two kinds of stress factors: mycotoxin and drought, respectively. Both the quantity and the spectrum of alkaloids were measured after different separation procedures. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used. TLC has the advantage of simplicity, low cost, minimum need of work in sample preparation and high sample throughput. HPLC is particularly well suited for the detection of very small quantities (ng) of alkaloids in the sample (Cikalo *et al.* 1992). The main question of the research is whether the quantity of synthesised alkaloids in poppy is influenced by the stress factors.

MATERIAL AND METHODS

Plant material: Papaver somniferum (cv. 'Kék Duna', Budakalász) plants were grown for 2 month from seeds in quartz-sand (in natural light, temperature: 24–28 °C, in Knop's nutritive solution).

Stress effects were the mycotoxin stress and drought stress. In the case of mycotoxin stress the leaves were sprayed with different concentration of fusaric acid solutions (0.001%, 0.01%, 0.1%) which was obtained from Sigma and we waited for 5 days. The other stress effect was the drought stress. We withdrew the water supply for 5 days.

Extraction, thin layer chromatography and densitometry: The extracts were concentrated by evaporation under vacuum (Büchi distillation apparatus) at 40 °C then these extracts were concentrated with 5 ml methanol again and was dissolved with 500 µl methanol. Extracts of *Papaver somniferum* (20 µl) were applied in the form of a band (5 mm) onto the layer (10 × 10) and the separation distance was 60 mm. Mobile phase was toluol-aceton-ethanol-ammonium-hidroxid (25%) (40:40:6:2). Development was performed in a glass TLC twin chamber (Camag) at ambient temperature. The plates were dried also at ambient temperature. Detection was performed by the use of the formaldehyde-sulphuric acid-reagent (Marquis' reagent). The plates were scanned for emitted light at $\lambda = 600$ nm, exciting the fluorescent derivatives with the 334 nm line of the mercury light source of the densitometer (CAMAG TLC SCANNER II), filter position was 400 nm.

HPLC separation: The broad spectrum HPLC analyser with coupled column system (REMEDi HS™ Drug Profiling System, BIO-RAD) using liquid chromatography with on-line sample analysis. A multicolumn approach is used to extract, purify and analyse drugs primarily in urine followed by multi-

wavelength UV-detection. As each drug enters the detector from the last cartridge, a UV scan from 200 nm to 300 nm is made. Sample spectra are then automatically compared with the library of known drug spectra stored in the PC's memory. This, in conjunction with chromatographic data, results in the identification of the drug.

Experiment with poppy plants: 1 g plantlet was rubbed with 30 ml methanol then it was turbo extracted (POLYTRON Turbo Extractor). The extract was filtered through filter paper (Siltrak). Five ml distilled water and 2 drops of concentrated HCl were added to the filtrate to make the pH acidic. Further concentration was done to reach 5 ml volume with the Büchi distillation apparatus. After filtration again the extract was completed to 5 ml with distilled water.

We mixed 1 ml of the extracts with 200 µl internal standard solution (BIO-RAD standards: Nordiazepam, N-ethyl and Chloropheniramine) and after centrifugation they were placed them into the apparatus.

Method of experiments HCHO content: We planted 0.2 g seeds of *Papaver somniferum* in 420 g soil in each flower pot. Eight pots were prepared. The water supply varied for 3 days (0 ml, 20 ml, 40 ml, 80 ml). 0.25 g whole plants were processed by each amount of water supply. Plants were freeze-dried, powdered and treated with 0.7 ml dimedone solution (0.2%, 0.5%, 1% concentration). The samples stood for 24 hours in a dark place after they were deposited. The clear extracts were studied by TLC (chloroform-methylene chloride, 35:65, v/v). We applied 25 µl from samples and from dimedone solution, we applied 2 µl formaldimedone on the thin layer. The quantity of HCHO (in dimedone adduct form) was determined by a densitometer (CAMAG TLC SCANNER II) (Tyihák *et al.* 1993).

RESULTS

Effect of drought stress

Papaver somniferum can be cultivated easily and it has about 50 alkaloids that can be determined with simple methods. Two kinds of methods were used in the determination of the alkaloids. The HPTLC method of detection does not eliminate all of the interfering substances but our measurements suggest that HPTLC might be a proper technique, because an adequate load of these compounds can be separated when applying this technique. The other method is the most sensitive and helps with the correct identification of the alkaloid spectrum. The broad spectrum HPLC analyser with coupled column system (REMEDi HS™ Drug Profiling System, BIO-RAD) uses liquid chromatography with on-line sample analysis. A multicolumn approach is used to ex-

Table 1
Measurement results of extracts of *Papaver somniferum* with REMEDi HSTM. Concentration (in ng/ml) = (R.F.) × Peak Height (uAU)

Alkaloid	Control (μg/ml)	Drought stress (μg/ml)
Morphine	7.47	31.60
Codeine	39.32	46.15
Narkotine	4.84	19.12

R.F. (morphine) : 0.013; R.F. (codeine) : 0.0149; R.F.: Response Factor: The drug list shows response factors which may be used in the semi-quantitative estimation of concentration using the previous formula

tract, purify and analyse drugs, primarily in urine, followed by multi-wavelength UV-detection. As each drug enters the detector from the last cartridge, an UV scan from 200 nm to 300 nm is made. Sample spectra are then automatically compared with the library of known drug spectra stored in the PC's memory. This, in conjunction with chromatographic data, results in the identification of the drug. The whole procedure is completed in approximately 20 minutes as it is briefly described below.

The influence of drought stress on the alkaloid content was determined after 5 days of the withdrawal of the water supply. The leaves of the plants started to dry when they were still living. The plants also tolerated dryness, and the withdrawal of water lasted for a short period. However, we can compare the mass of the grown poppy plants. The mass of the control group was 36.2 g, while in case of drought stress the mass of plants was 24.7 g. So the process of drooping had started by then.

Compared of the control group, three types of alkaloids (morphine, codeine, narkotine) could be measured in the extracts. Figure 1 shows the results of typical chromatograms. In the standard morphine alkaloid sample, narkotine, morphine and codeine were identified (Figure 1a). In Figure 1b the chromatogram of control plantlets is shown. Number 9 marks the narkotine alkaloid, number 19 marks the morphine alkaloid and number 21 marks the codeine alkaloid. These peaks show the quantity of alkaloids approximately, because this HPLC method is semiquantitative (with about 30% inaccuracy). In spite of all this procedure, we could differentiate between the observed groups. The Figure 1c shows the effect of drought stress. On the chromatogram it can be seen that these three sorts of alkaloids (narkotine, morphine, codeine) appear and peaks of narkotine and morphine are higher than in case of the control group. Table 1 indicates the results of the measurement of HPLC with REMEDi HS™. According to the table, we can see that the quantity of codeine is also higher than in the control group. The extracts of poppy plantlets had high alkaloid levels in case of the applied drought stress. The broad spectrum

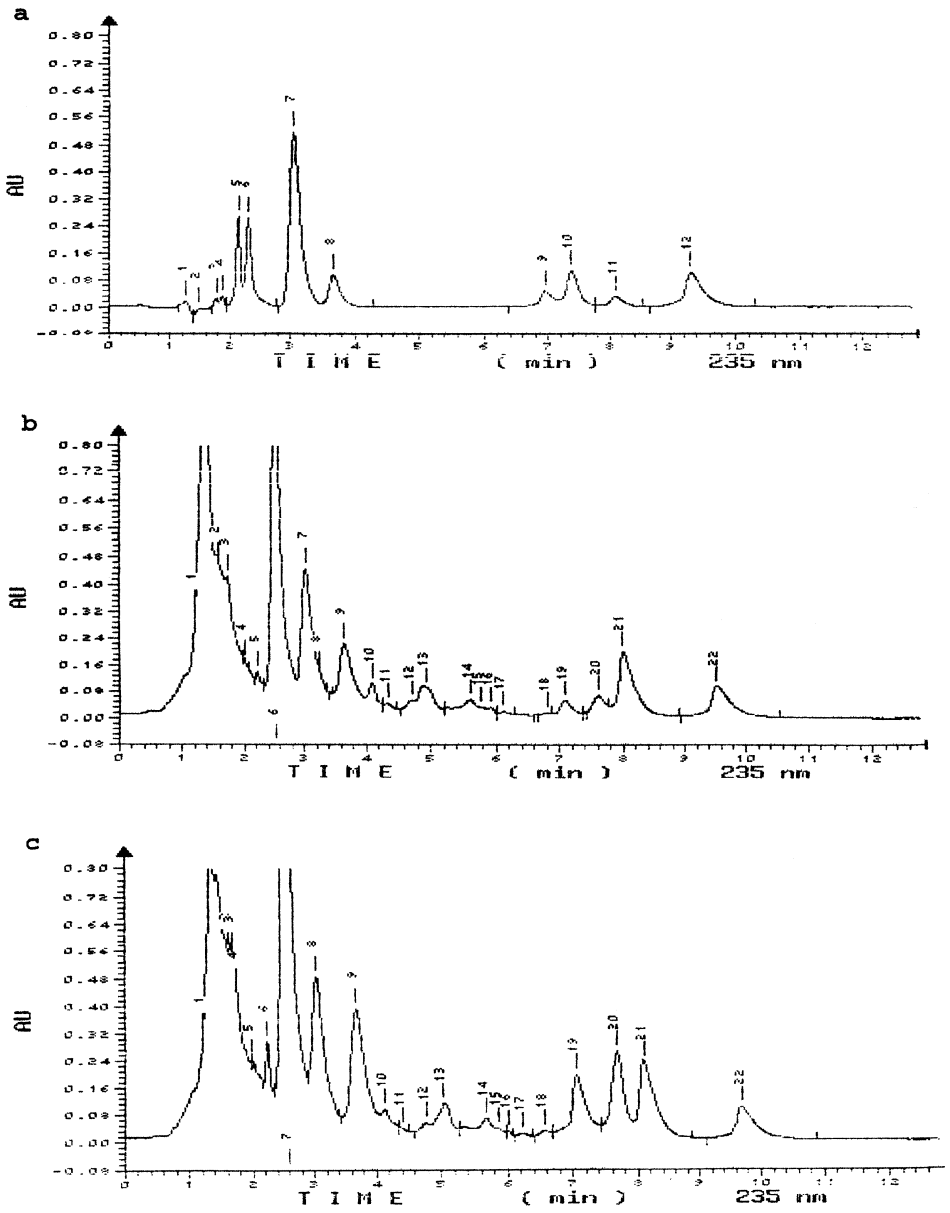

Fig. 1. Chromatograms of extracts of *Papaver somniferum* with REMEDi HS^{TM}. – a = Running of morphine alkaloids (6 = papaverine, 8 = narkotine, 9 = morphine, 10 = tebaine, 11 = codeine). – b = Extract of *Papaver somniferum* (control) (9 = narkotine, 19 = morphine, 21 = codeine). – c = Extract of *Papaver somniferum* after drought stress (9 = narkotine, 19 = morphine, 21 = codeine)

Table 2
Measurement results of extracts of *Papaver somniferum* with REMEDi HSTM. Concentration (in ng/ml) = (R.F.) × Peak Height (uAU)

Alkaloid	Control (µg/ml)	Fusaric acid effect (µg/ml)		
		0.1%	0.01%	0.001%
Morphine	7.47	6.91	9.01	5.92
Codeine	39.32	18.06	17.77	24.37
Narkotine	4.84	0	1.85	3.23

R.F. (morphine) : 0.013; R.F. (codeine) : 0.0149; R.F.: Response Factor: The drug list shows response factors which may be used in the semi-quantitative estimation of concentration using the previous formula

HPLC analysis is the most sensitive method, and we could identify the alkaloids correctly. Serious differences can be seen between the drought stress group and the control group in case of the morphine and narkotine. In the quantity of the codeine alkaloid significant discrepancy cannot be observed.

Effect of mycotoxin stress

In the case of mycotoxin stress the leaves were sprayed with a pulveriser containing different concentrations of fusaric acid solutions (0.001%, 0.01%, 0.1%). The mycotoxin solutions were obtained from Sigma, and the plants were put aside for 5 days. The morphology of plantlets did not indicate change. They were green and they did not have any spots or discolouration.

The data of Table 2 do not show considerable difference in case of the morphine alkaloid, while the measured results of the codeine and narkotine alkaloids show different items. Compared with the control group, we got lower quantity of alkaloids. We also have to prove these results have to be confirmed by another procedure. In this case, the question is whether the concentration of mycotoxin (fusaric acid solution) was low or the method of spraying allocated little solution to the leaves. In the future, another method could also be applied, in which the fusaric acid solution is added to the nutritive solution on the soil.

DISCUSSION

As a consequence, the stress effects can be detected in poppy plants by two kinds of method. At first we measured the content of alkaloids. Drought stress produced a higher level of the alkaloids, but the mycotoxin stress did not show significant results.

Earlier experiments indicate that measurable formaldehyde level is dramatically elevated in virus or other microbial infected plant tissues (biotic stress) and in the case of heat shock and salt stress (abiotic stress). At the same time, the level of different methylated compounds as potential HCHO generators, are considerably decreased. This involves that the alarm reaction phase of stress syndrome includes an intensive demethylation process (Tyihák *et al.* 1993). Formaldehyde (HCHO) is a normal constituent of poppy plantlets similar to other plant species (Adrian-Romero *et al.* 1999). HCHO can be detected in injured tissues of *Papaver somniferum*, this can be shown as a dimedon-adduct form with thin layer chromatography (Tyihák *et al.* 1981). So we tried to study the stress-effects in plants in a different way, for instance by measuring formaldehyde level in the samples (Tyihák *et al.* 1993.). We measured the level of formaldehyde, in the form of formaldimedone. In case of drought stress the highest level of HCHO was identified. It seems that different stress effects influence the level of alkaloids in the poppy plants.

The question is whether the methyl groups of different poppy alkaloids are potential HCHO generators and are suitable for the elimination of the stress injuries; and the formation of methyl groups of poppy alkaloids takes place through HCHO, similarly to the already known case of histamine.

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