Relations among alkaloids, cadmium and zinc contents in opium poppy (*Papaver somniferum* L.)

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ABSTRACT

The effort of this work was to prove whether there exists any relation between stress factors caused by content of cadmium and zinc, and content and composition of opium alkaloids codeine, morphine, narcotine (noscapine) and papaverine contained in poppy seeds and capsules (*Papaver somniferum* L.) in 14 samples of 8 varieties cultivated in 8 localities in 2003. Contents of cadmium and zinc were measured by a method of electrothermic atomization and contents of selected individual alkaloids were determined by a HPLC method. The results were tested by the Spearman correlation coefficient and the Pearson linear correlation coefficient. With knowledge of the Spearman coefficient, positive relations narcotine – zinc and narcotine – cadmium in the poppy seed samples were found. In the samples of poppy capsules positive relation morphine – cadmium (in cv. Opal) and negative relation papaverine – zinc and codeine – zinc (in cv. Opal) were found. Those pairs have been tested by the Pearson correlation coefficient for a possibility to exclude the linear independency. This independency was excluded with the probability of over 95% in the relations narcotine – cadmium in the seeds and morphine – cadmium in the capsules. With the probability of over 97.5%, linear relations narcotine – zinc in seeds and papaverine – zinc in capsules were proved.

Keywords: *Papaver somniferum* L.; poppy capsules; poppy seeds; cadmium; zinc; stress; alkaloids; codeine; morphine; narcotine (noscapine); papaverine

Poppy (*Papaver somniferum* L.) produces secondary metabolites, which have important roles in self-defence processes – alkaloids (Szabo et al. 2003). Pothier and Goland (2005) described as major alkaloids contained in poppy morphine, codeine, thebaine, papaverine, narcotine (noscapine). Poppy capsules with a high alkaloid content are of great importance to the pharmaceutical industry (Szucs et al. 2002). On the contrary, a mutation-breeding programme using γ-rays and ethyl methane sulphonate was carried out for genetic conversion of narcotic opium poppy into non-narcotic seed poppy (Sharma et al. 1999). Poppy, despite the low Cd concentrations in the soil, reaches the highest Cd concentrations in the seeds (Chizzola 1997). Chizzola et al. (2003), Tlustoš et al. (1997) and Pavlíková et al. (1997) confirmed a high tendency of the poppy to accumulate heavy metals, above all Cd and Pb and also semi-metal arsenic. As Pavlíková et al. (1997) found, spraying of poppy during the growing season by potassium humate resulted in a significant growth of Cd and Zn contents in the plant. The concentration of alkaloids in capsules is affected by many factors. Kadar et al. (2001) reported an increase of alkaloids in capsules as the result of N fertilisation, while it generally decreased following P fertilisation. Also irregular stress effects change the alkaloid content of poppies. Szabo et al. (2003) observed that drought stress resulted in higher levels of the alkaloids whereas mycotoxin stress did not result in significant differences. As reported by Morimoto et al. (2001), in response to stress, morphine is quickly metabolised to bismorphine consisting of two morphine units. Bismorphine binds predominantly to pectins possessing high galacturonic acid residue contents through ionical bonds with higher ability as Ca^{2+} – a cross-linker

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of these polysaccharides. Bismorphine could be so evaluated as a defence response of the opium poppy. Morimoto et al. (2003) together with bismorphine A identified also bismorphine B in the wounded capsules of *Papaver somniferum*.

Regarding this knowledge, the aim of the this study was to determine if abiotic stress caused by higher concentrations of Cd and Zn could affect the content of individual poppy alkaloids and if there exist some relationships between them.

MATERIAL AND METHODS

Plant material and sampling. For the determination of alkaloid, Cd and Zn contents, 14 samples of 8 varieties of poppy (*Papaver somniferum* L.) from 8 different localities of the Czech Republic from the harvest in 2003 were used (Table 1). In the year 2003 the deviation of air temperature from long-term normal 1961–1990 in the Czech Republic was +0.9°C and mean precipitation amount (expressed as percentage of the long-term normal 1961–1990) was 77%. From this aspect, the year 2003 could be evaluated as mean in temperature and drier as compared to the long-term normal. The localities, on which the crop was cultivated, were mainly situated in areas with mean above sea level 250–290 m, with the exception of higher situated Červený Újezd (593 m a.s.) and Žďár nad Sázavou (580 m a.s.) localities. Lower situated localities were Hulín (191 m a.s.) and Pravčice (193 m a.s.) or Prostějov (225 m a.s.). In the lower situated localities prevail fertile predominantly loam soils (Orthic Luvisol and black Luvic Chernozem), in higher localities less fertile predominantly sandy loam soils (prevails Cambisol). All analysed varieties belong to opium seed varieties with blue coloured seeds. Hanácký modrý variety distinguishes with higher oil content, new introduced varieties Major, Malsar and Maraton give high seed yield and with Lazur variety also higher morphine contents. Samples were harvested by manual way of harvest in the phase of harvest maturity. Capsules were dried to dryness and stored till the analyses by means of cold air and aeration tubes. Capsules and seeds were separated immediately before analyses. Alkaloid, Cd and Zn contents were determined in dry material and expressed in dry matter.

Table 1. Poppy varieties and localities of their cultivation

No. of sample	Variety	Locality	Above sea level (m)
1	Gerlach	Dřetovice	270
2	Hanácký modrý	Dřetovice	270
3	Lazur	Holasovice	278
4	Major	Dřetovice	270
5	Malsar	Dřetovice	270
6	Maraton	Dřetovice	270
7	Opal (1)	Hulín	191
8	Opal (2)	Červený Újezd	593
9	Opal (3)	Pravčice	193
10	Opal (4)	Žďár nad Sázavou	580
11	Opal (5)	Dřetovice	270
12	Opal (6)	Prostějov	225
13	Opal (7)	Jaroměř	250
14	Zeno	Dřetovice	270

Isolation of alkaloids. Capsules (not attacked by diseases) and poppy seeds were powdered in an electrical grinder into little particles. Samples (about 10 g) were extracted in a Soxhlet apparatus with 150 ml of methanol for 24 hours. After evaporation in a vacuum rotational evaporator, the alkaloids were extracted from the total solids three times with 10 ml of 3% H_2SO_4. All three extracts were combined, filtered and then twice extracted with 15 ml of diethyl ether. After separation in a separation funnel, water layer was alkalised by addition of NH_4OH and then twice agitated with 15 ml of chloroform. Chloroform portions were combined and dried with Na_2SO_4. After filtration, the samples were evaporated in the vacuum rotational evaporator to dryness and the solids were solved in 10 ml of methanol.

HPLC analysis of alkaloids. Analyses were performed using a liquid chromatograph Waters^{TM}, pump Waters^{TM} 615, automatic sampler Waters^{TM} 717 plus, diode array detector PDA Waters^{TM} 966 UV-VIS. Alkaloids were separated by isocratic elution on Zorbax Extend-C 18 column (3 × 250 mm, i.d. 5 µm) with mobile phase methanol – buffer 0.025M Na_2HPO_4 + 0.025M NaH_2PO_4) in volume ratios 1:1/55:45. Chromatographic parameters were: flow rate 0.5 ml/min, injection 10 µl, column temperature 35°C, pressure 14 479 kPa, detection at wave lengths λ = 215 nm and λ = 254 nm. For the determination of the contents of the individual alkaloids, standard solutions of morphine, codeine and narcotine were used (concentrations of standard solutions were 1 mg/ml, 0.1 mg/ml and

0.01 mg/ml), minimum linear calibration range was 0–1.5 µg/ml. Average results were obtained from three parallel determinations. Relative standard deviations of the determinations were 3–12%.

Determination of Cd and Zn. Capsules and seeds were analysed by AAS method. Dry samples (1 g) were decomposed by a method of dry decomposition, i.e. charring on hot plate in the temperature range of 180–360°C and then by ashing in an electrical muffle furnace at temperature range 350–500°C. Undecomposed residues of organic character were oxidised by concentrated HNO_3 and decomposed at 500°C. Ash was diluted in 1.5% HNO_3 and dissolution was accelerated by sonication (Mader et al. 1998). Cd content was measured by electrothermic atomisation (ET-AAS) using Varian SpectrAA 400 spectrometer with graphite atomiser GTA-96 and Zn content with flame technique (F-AAS) using Varian SpectrAA 110 spectrometer with SIPS sampler. Level of the background of laboratory was measured by the analyses of blank samples and quality of analytical data was proved by parallel analyses of an internal reference material. Quality of the analytical data was assessed by parallel analysis of the experimental samples with the samples with known content of the analytes determined. Those were IPE 949 Aubergine and IPE 950 Melon obtained from an inter-laboratory test IPE (WEPAL, NL, Table 4). Obtained results were above limits of detection (LOD) (for Cd 0.01 µg/l and 0.009 mg/l for Zn), average relative standard deviation (RSD) for Cd was 2.6% and for Zn 1.75%.

Statistic evaluation. The obtained results of three parallel determinations were evaluated by Excell and Statcrunch 4.0 beta programmes. For the evaluation of results, methods using Pearson linear correlation coefficient and Spearman correlation coefficient were applied. Pearson linear correlation coefficient was calculated as

$$r_p = \frac{\frac{1}{N} \times \sum x_i \times y_i - \bar{x} \times \bar{y}}{\sqrt{\frac{1}{N} \times \sum x_i^2 - \bar{x}^{\,2}} \times \sqrt{\frac{1}{N} \sum y_i^2 - \bar{y}^{\,2}}}$$

where: x represents Cd or Zn content, *y* alkaloid content and *N* is a number of value pairs

Spearman coefficient was expressed as

$$r_s = 1 - \frac{6\sum_{i=1}^{n} \left(\mathrm{rank}\,(X_i) - \mathrm{rank}\,(Y_i)\right)^2}{n\left(n^2 - 1\right)}$$

RESULTS AND DISCUSSION

Mean alkaloids, Cd and Zn contents in poppy samples are given in Table 2. As results from the data, in both poppy capsules and seeds content of analysed alkaloids decreased in order morphine > papaverine > codeine > narcotine. The content of the alkaloids in the capsules was many times higher in comparison with their content in seeds. As Shukla and Singh (2001) confirmed, the highest morphine, codeine and thebaine contents were determined in capsules and maximum morphine content in capsules was reached at maturity stage. The highest contents were found in varieties Hanácký modrý, Zeno and Opal, whereas cv. Major, Maraton and Malsar contained lower levels of alkaloids, confirming thus significant differences among varieties reported by Sharma et al. (1999). As could be demonstrated on the Opal variety with 7 analysed samples, alkaloid content depends also on locality, where it was cultivated (Dřetovice, Jaroměř × Pravčice). Cd and Zn contents were lesser in capsules in comparison with seeds, which is in good agreement with the results obtained by Chizzola (1997, 2001) or Balík et al. (1998). As Chizzola (2001) reported, seeds made up 2.5% to 12.9% of above-ground biomass, but stored 15 to 42% of total Cd, which indicates a preferential translocation of Cd into seeds. Content of zinc was much more higher than the cadmium content in both capsules and seeds. Moreover, Infante et al. (1999) detected in poppy drug a lower content of copper and iron, which can increase the inherent toxicity.

Spearman and Pearson correlation coefficients for the pairs between the individual alkaloids and Cd or Zn contents from the results of all varieties and Opal variety are given in Table 3. Higher Cd content caused an increase and activation of biosynthesis of narcotine in seeds (Figure 1) of all varieties and capsules of Opal variety as well as of morphine in capsules of cv. Opal. Cadmium is toxic for plants and induces an abiotic stress. Poppy plants response to the stress by an increase of alkaloid content, namely of narcotine, morphine and papaverine in capsules of cv. Opal (Table 3). On contrary, higher Zn content resulted in a decrease of narcotine in the seeds of all varieties (Figure 3), codeine in Opal variety capsules, and of papaverine (Figure 2) and narcotine in capsules of all varieties (Table 3). Zinc is a microbiogenic element for the poppy plant and its higher content could be correlated with lower content of major alkaloids, namely of codeine, papaverine and narcotine in capsules.

Table 2. Mean opium alkaloids, Cd and Zn contents in poppy samples (mg/kg dry matter)

Poppy part	Sample	Morphine	Codeine	Papaverine	Narcotine	Cd	Zn
Seeds	Gerlach	2.33*	0.46	0.41	0.09	0.48 ± 0.07	61.1 ± 1.42
	Hanácký modrý	15.5	2.55	4.03	2.73	0.51 ± 0.01	69.7 ± 0.30
	Lazur	4.87	2.27	25.1	0.79	0.73 ± 0.04	83.4 ± 1.29
	Major	0.82	0.34	2.34	0.08	0.27 ± 0.01	57.3 ± 0.23
	Malsar	1.92	0.66	traces	0.05	0.20 ± 0.01	60.0 ± 0.51
	Maraton	1.33	0.41	1.45	0.07	0.29 ± 0.01	58.7 ± 0.59
	Opal (1)	0.69	1.12	1.28	0.11	0.71 ± 0.08	71.5 ± 0.38
	Opal (2)	0.13	0.75	0.76	0.18	0.71 ± 0.13	78.3 ± 0.17
	Opal (3)	1.05	2.04	0.60	1.34	0.92 ± 0.18	79.7 ± 0.74
	Opal (4)	0.71	0.43	0.83	1.16	1.17 ± 0.09	94.3 ± 1.52
	Opal (5)	1.77	1.16	0.88	0.37	0.35 ± 0.03	82.3 ± 1.00
	Opal (6)	1.68	0.27	0.54	0.28	0.41 ± 0.05	67.8 ± 0.60
	Opal (7)	0.35	traces	0.31	2.60	0.74 ± 0.04	90.3 ± 0.19
	Zeno	13.2	3.40	4.03	0.39	0.33 ± 0.01	82.5 ± 2.45
	mean	3.31	1.13	3.04	0.73	0.56 ± 0.05	78.1 ± 0.81
Capsules	Gerlach	1381	270	1169	166	0.07 ± 0.04	5.76 ± 0.12
	Hanácký modrý	2599	611	506	1352	0.17 ± 0.02	6.92 ± 0.16
	Lazur	582	430	826	205	0.21 ± 0.02	5.91 ± 0.21
	Major	1283	340	1671	199	0.07 ± 0.05	4.98 ± 0.20
	Malsar	1830	314	1275	242	0.08 ± 0.09	5.83 ± 0.27
	Maraton	955	167	493	55.9	0.07 ± 0.05	5.91 ± 0.56
	Opal (1)	2319	325	588	119	0.42 ± 0.10	14.6 ± 0.28
	Opal (2)	1919	407	35.3	42.1	0.37 ± 0.04	15.6 ± 0.24
	Opal (3)	2727	399	1279	181	1.09 ± 0.16	12.7 ± 0.59
	Opal (4)	3366	349	511	113	0.44 ± 0.13	16.5 ± 0.51
	Opal (5)	595	636	756	41.8	0.16 ± 0.04	8.22 ± 0.09
	Opal (6)	698	194	1246	198	0.44 ± 0.07	15.2 ± 0.50
	Opal (7)	536	232	35.0	6.03	0.27 ± 0.01	37.4 ± 1.35
	Zeno	2603	617	1190	125	0.58 ± 0.33	8.42 ± 0.72
	mean	1671	378	827	218	0.32 ± 0.08	11.7 ± 0.41

**RSD* of HPLC determination of poppy alkaloids was in the range 3–12%

Enhanced biosynthesis, or reversely, decrease of alkaloid contents in poppy capsules and seeds regarding the Cd and Zn levels, could also be related to ability to form chelate complexes with hydroxyl groups (molecule of morphine contains two hydroxyl groups). Also their precursors containing in their structure *o*-dihydroxy groups could be involved in this process. In biosynthesis, dopamine and 3,4-dihydroxyfenylpyruvic acid are key precursors of papaverine, while L-DOPA and dopamine of morphine, and (*S*)-norcoclaurine and (*R*)-reticuline of codeine. The constitutive presence of morphine, together with bismorphine and metabolites involved in poppy alkaloid biosynthesis could be in this way involved in inducing the defence system what is in a good agreement with conclusions of Morimoto et al. (2001) and results obtained by Szabo et al. (2003).

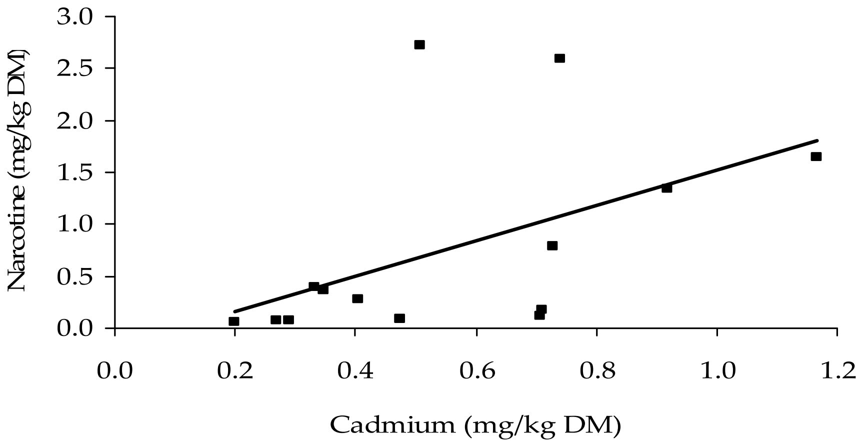

Figure 1. Linear relationship narcotine – cadmium in poppy seeds

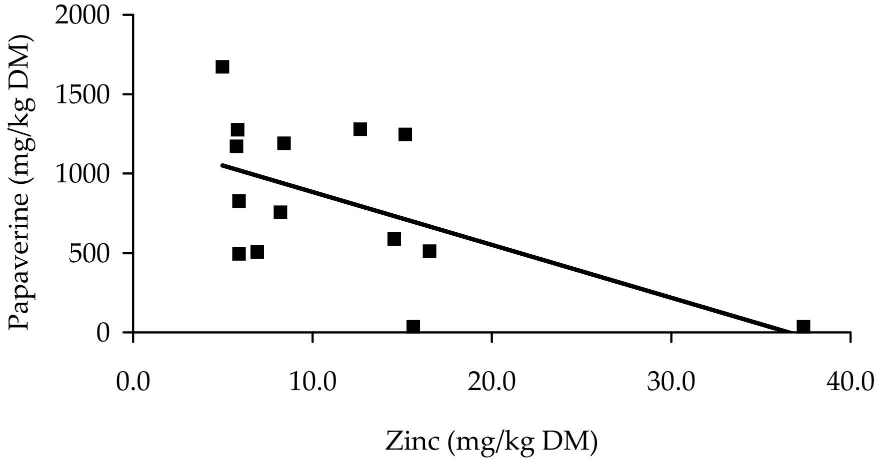

Figure 2. Linear relationship papaverine – zinc in poppy capsules

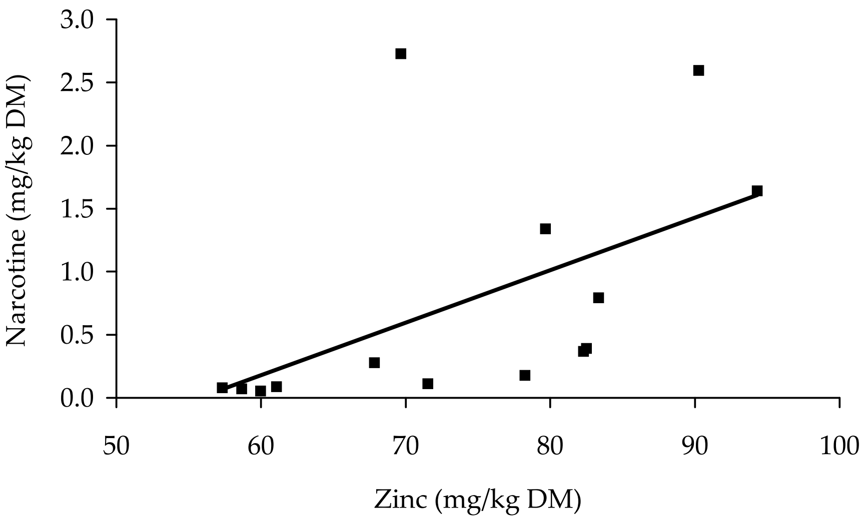

Figure 3. Linear relationship narcotine – zinc in poppy seeds

By means of Spearman correlation and Pearson linear correlation coefficients, positive linear relations were proved between narcotine – cadmium and narcotine – zinc in seeds and morphine – cadmium (esp. in cv. Opal) in capsules. Cadmium in higher concentrations caused abiotic stress and

Table 3. Spearman (r_s) and Pearson (r_p) correlation coefficients

Poppy part	Pair	All varieties		Opal variety	
		r_s	r_p	r_s	r_p
Seeds	morphine-Cd	–0.3758	–0.2247	–0.4286	–0.5873*
	morphine-Zn	–0.1253	0.0343	–0.0714	–0.3617
	codeine-Cd	0.0857	0.0072	–0.1071	0.0851
	codeine-Zn	0.2791	0.2463	–0.1071	–0.2411
	papaverine-Cd	–0.0681	0.1250	–0.2500	0.0185
	papaverine-Zn	0.0945	0.2088	–0.0357	–0.2805
	narcotine-Cd	0.7275**	0.5053	0.5714**	**0.5388***
	narcotine-Zn	0.7714**	0.5345*	0.8214**	**0.7795***
Capsules	morphine-Cd	0.4945	0.5108	0.8214**	**0.5610***
	morphine-Zn	0.1648	–0.0992	–0.0714	–0.3014
	codeine-Cd	0.2615	0.1398	–0.2500	–0.1286
	codeine-Zn	–0.0242	–0.3053	–0.5000	–0.6036*
	papaverine-Cd	0.0022	0.0917	0.5000	0.5847*
	papaverine-Zn	–0.5209	–0.5760*	–0.7857**	**–0.5713***
	narcotine-Cd	–0.1473	–0.1351	0.7857**	**0.6767***
	narcotine-Zn	–0.5868**	–0.2753	–0.3214	–0.4641

*statistically significant Pearson correlation coefficients (r_p) for n = 14, level of significance α = 0.05 and critical value 0.5324

**statistically significant Spearman correlation coefficients (r_s) for n = 14, level of significance α = 0.05 and critical value of Spearman correlation coefficient 0.5341

Table 4. Assessment of obtained results by parallel analysis of samples obtained from an inter-laboratory test IPE (WEPAL, NL)

IPE WEPAL NL	Cd_{found} (mg/kg dry matter)	$Cd_{known\ from\ IPE\ test}$ (mg/kg dry matter)
IPE 949 Aubergine WEPAL NL	0.448 ± 0.024	0.421 ± 0.033
IPE 950 Melon WEPAL NL	1.073 ± 0.067	1.028 ± 0.100
IPE WEPAL NL	Zn_{found} (mg/kg dry matter)	$Zn_{known\ from\ IPE\ test}$ (mg/kg dry matter)
IPE 949 Aubergine WEPAL NL	102 ± 1	107 ± 7
IPE 950 Melon WEPAL NL	84.4 ± 1.3	80.9 ± 4.6

enhancing of the content of alkaloids could be involved in poppy defence system. While in the seeds positive relation between narcotine and zinc was found, negative relations between papaverine-zinc and codeine-zinc (noticeably in cv. Opal) prevailed in the capsules. This fact could be related to the positive role of zinc in poppy protein and saccharide metabolism and growth.

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