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Variability of alkaloid content in *Papaver somniferum* L.

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Summary

A total of 300 accessions of opium poppy (*Papaver somniferum* L., Papaveraceae) of the IPK genebank collection from nearly all over the world were cultivated under field conditions in Gatersleben for morphological and phytochemical characterisation. Altogether 35 morphological and agronomic characters were collected for all accessions. Determination of chromosome numbers with flow cytometry showed that the accessions of subspecies *setigerum* are tetraploid whereas all accessions of the other subspecies are diploid.
Composition and content of the five main alkaloids (morphine, codeine, thebaine, papaverine and noscapine) were analysed by high performance liquid chromatography (HPLC). Total alkaloid content varied between 683.32 and 25,034.84 µg/g dry matter (first year) and 1,799.49 and 25,338.55 µg/g dry matter in the second year of cultivation. There is a highly significant correlation between total content of alkaloids and morphine in both years (r=0.926/P=0.000; r=0.918/P=0.000). In contrast, the other four main alkaloids show less or no correlation with each other or the total alkaloid content. This analysis demonstrated that the amount and composition of the main alkaloids are highly variable. Additionally, there is no important correlation between morphological characters and alkaloid content. So it is not possible to use these characters as a prediction tool of alkaloid content during breeding process.

Introduction

Papaver somniferum L. is one of the few species which has been used since the Neolithic. Its worldwide distribution results from its long history in cultivation. The use of poppy alkaloids for medicinal purpose is proved for the Middle Ages, when it was an ingredient of the tincture called 'theriak'. Later in the 16^{th} century Paracelsus developed another famous tincture called 'laudanum' (SEEFELDER, 1996). Opium poppy contains more than 40 different alkaloids. The five main alkaloids of *P. somniferum* are morphine, codeine, thebaine, papaverine and noscapine (DINGERMANN et al., 2004). Morphine is the dominant alkaloid and a strong naturally occurring pain reliever. An industrial synthesis of this substance is possible but with very low yield, e.g. 10% in Fuchs-Synthesis. Codeine and noscapine are used as cough suppressant and papaverine is a smooth muscle relaxant. Thebaine is not directly used therapeutically but is industrially converted into other pain relievers.

During the long time of cultivation a lot of different forms (landraces, cultivars, varieties and chemical types) which belong to one species have been bred and cultivated. The infraspecific classifications of DANERT (1958) and HAMMER (1981) as well as HANELT and HAMMER (1987) are based on a few morphological characters such as capsule dehiscence, shape of stigmatic lobes and colour of flowers and seeds. It is differentiated between three subspecies (*setigerum* (DC.) Corb., *somniferum* and *songaricum* Basil.), four convarieties and a total of 52 botanical varieties. The subsp. *setigerum*, separated from the other two subspecies according to the hairiness of buds, is supposed to be the wild relative of the cultivated subspecies (HAMMER & FRITSCH, 1977; KADEREIT, 1986).

Actually there exist two contrary breeding aims: the first breeding for lines with a very high content of alkaloids for medicinal purpose and the opposite aim are lines with very low alkaloid content for food production (PRAJAPATI et al., 2002). The aims of this work were at first to investigate the variability of morphological characters as well as the variability of alkaloid content. The second point is to find out if there is a correlation between morphological and phytochemical data and if it is possible to use special morphological characters as a prediction tool of alkaloid content during breeding process.

Materials and methods

Plant material and morphological characterisation

In total 300 accessions of *P. somniferum* from nearly all over the world were cultivated under field conditions in Gatersleben in 2005, 2006 and 2007. The morphological characterisation was done according to a descriptor (DITTBRENNER et al., 2008). This descriptor was developed to standardise the characterisation and contains chromosome number as well as agronomic and morphological characters including flowering date, plant height, number and size of capsules, shape and hairiness of leaves, as well as flower and seed colour.

During the whole time of field cultivation weather conditions for example temperature, amount of rainfall and solar radiation were measured. The amount of rainfall in the period of time from flowering until the end of maturation was higher in 2005 with 159.9 mm compared to 123.1 mm in 2006 and 247.4 mm in 2007. These values were accompanied by lower temperatures in 2005. In this year an average of 23.53 °C was measured 2 m above ground compared to 25.62 °C in 2006 and 24.22 °C in 2007.

Phytochemical analyses

Ripe and dried capsules from the first flower of a plant (primary capsule) were harvested in the field from all 300 accessions in the years 2005 and 2006. In 2007 only a selection of 40 accessions which represented 10 accessions with very low alkaloid content, 10 with the highest and 20 accessions from the middle were measured to validate results from the other two years. Seeds, stigmatic disc and stalk were removed. Nine capsules per accession were ground to powder. For methanol extraction (75% MeOH) 50 mg of a mixture of three capsules were used. In total three pooled samples per accession were analysed. Extraction took place in an ultrasonic bath at 40 °C. The extracts were resolved by reversed phase HPLC (Agilent System). Columns were 150 x 2.00 mm packed with 5 µm porous silica gel (C18 Gemini; phenomenex). A gradient analysis (Tab. 1) was carried out with the following solvents: a mixture of 0.1 N NH_3 solution and 0.1 N NH_4Cl solution with a pH of 8.8 (A) and 100% acetonitrile (B). The flow rate was 0.2 ml/min and injection volume 10 µl. For peak identification the retention time as well as the UV-Vis spectrum in the range of 200-400 nm of each substance were used. The content of the five main alkaloids (morphine, codeine,

Tab. 1: HPLC gradient for separation of alkaloids. (Solvent A: a mixture of 0.1 N NH_3 solution and 0.1 N NH_4Cl solution; solvent B: acetonitrile)

minutes	% A	% B
0	76	24
11	76	24
14	64	36
33	29	71
36	14	86
46	0	100
50	0	100
60	76	24
75	76	24

thebaine, papaverine and noscapine) was calculated in µg/g dry matter (DM) in reference to the calibration curve of each main alkaloid at 280 nm. The Empower software was used for data acquisition and analysis. Statistical analysis was done with SPSS 10.0 (1999). For metric/non metric data Pearson's/Spearman's correlations coefficient were used for correlation analyses between morphological characters and content of alkaloids determined in 2005 and 2006.

Results

Phytochemical analyses

A high variation could be detected with respect to the quantitave composition of the main alkaloids within all 300 accessions. The morphine content ranged from 363.00 to 17,749.05 µg/g dry matter in the year 2005, from 978.10 to 22,575.20 µg/g dry matter in 2006 and from 573.30 to 13,554.95 µg/g dry matter in 2007 (Tab. 2). There was a highly significant correlation between total content of alkaloids and morphine in both years (2005: r=0.926/P=0.000; 2006 (Tab. 4): r=0.918/P=0.000). In 2007 a higher correlation coefficient between total content of alkaloids and morphine (r=0.963/P=0.000) could be detected within the 40 accessions. In nearly all accessions morphine was the main alkaloid. In 2005 there were just six accessions with a different main alkaloid: two with papaverine, two with codeine, one with thebaine and one with noscapine as the most abundant alkaloid. In the second year three accessions showed a higher content of papaverine than morphine. Within the analysis of the 40 accessions of 2007 there were again three accessions with higher papaverine content than morphine and one accession in which noscapine was the most abundant alkaloid. The bar diagram (Fig. 1) demonstrates total alkaloid content in both years for a selection of 10 accessions which represents a cross section through the profile of all 300 accessions. Some accessions like M408, M670 and M691 had a clearly increased content in 2006 compared with 2005 while M238 and M755 had lower values in 2006. A similar picture could be shown for comparison of morphine content in both years (Fig. 2). The composition of the main alkaloids varied from accession to accession and between the years 2005 and 2006 (Fig. 3, 4). Some lines like M408 and M691 had a clearly increased content of noscapine in 2006 compared to 2005, while the codeine content of M238 is decreased in the second year of cultivation compared to 2005. The t-test showed that within all 300 accessions values for morphine, noscapine and thebaine are significantly different and in most cases higher in the second year of cultivation compared to 2005. This behaviour could not be detected for codeine and papaverine, respectively.

Tab. 2: Range of main alkaloids and total alkaloid content measured during three years. (DM - dry matter).

	content 2005 (µg/g DM)		content 2006 (µg/g DM)		content 2007 (µg/g DM)	
alkaloids	**minimum**	**maximum**	**minimum**	**maximum**	**minimum**	**maximum**
codeine	6.01	3,595.13	3.00	2,718.39	37.82	2,827.96
morphine	363.00	17,749.05	978.10	22,575.20	573.30	13,554.95
noscapine	0.00	5,947.52	0.00	6,641.89	0.00	6,637.92
papaverine	0.00	3,151.21	0.00	2,817.48	3.79	3,607.48
thebaine	0.02	2,797.36	0.35	2,942.29	1.27	2,574.87
total	683.32	25,034.84	1,609.49	25,338.55	1,126.21	18,749.14

Tab. 3: Spearman's correlations coefficient between content of main alkaloids and some morphological characters. (C-codeine, M-morphine, N-noscapine, P-papaverine, T-thebaine)

		C	**M**	**N**	**P**	**T**	**total content**
plant colour	Corr. Coeff.	0.262	0.228	0.225	0.456	0.171	0.348
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.003	0.000
anthocyanins	Corr. Coeff.	-0.137	-0.076	-0.144	-0.417	-0.046	-0.191
in buds	Sig. (2-tailed)	0.018	0.191	0.013	0.000	0.428	0.001
flower colour	Corr. Coeff.	0.140	0.179	0.180	0.474	0.084	0.291
	Sig. (2-tailed)	0.017	0.002	0.002	0.000	0.153	0.000
colour	Corr. Coeff.	0.096	-0.088	-0.012	0.323	-0.081	0.005
of filaments	Sig. (2-tailed)	0.100	0.134	0.836	0.000	0.165	0.935
capsule	Corr. Coeff.	0.007	0.229	0.111	0.458	-0.010	0.270
dehiscence	Sig. (2-tailed)	0.902	0.000	0.061	0.000	0.872	0.000

Tab. 4: Pearson's correlations coefficient between content of main alkaloids and two morphological characters. (C-codeine, M-morphine, N-noscapine, P-papaverine, T-thebaine)

		C	M	N	P	T	total content
codeine	Corr. Coeff.	1	0.231	0.232	0.284	0.480	0.432
	Sig. (2-tailed)		0.000	0.000	0.000	0.000	0.000
morphine	Corr. Coeff.	0.231	1	0.208	0.163	0.207	0.918
	Sig. (2-tailed)	0.000		0.000	0.005	0.000	0.000
noscapine	Corr. Coeff.	0.232	0.208	1	0.368	0.031	0.506
	Sig. (2-tailed)	0.000	0.000		0.000	0.588	0.000
papaverine	Corr. Coeff.	0.284	0.163	0.368	1	0.108	0.407
	Sig. (2-tailed)	0.000	0.005	0.000		0.063	0.000
thebaine	Corr. Coeff.	0.480	0.207	0.031	0.108	1	0.342
	Sig. (2-tailed)	0.000	0.000	0.588	0.063		0.000
total content	Corr. Coeff.	0.432	0.918	0.506	0.407	0.342	1
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	
plant height	Corr. Coeff.	-0.178	-0.117	-0.088	-0.399	-0.025	-0.193
	Sig. (2-tailed)	0.002	0.043	0.130	0.000	0.672	0.001
seed yield	Corr. Coeff.	-0.163	-0.195	-0.207	-0.459	-0.096	-0.304
per capsule	Sig. (2-tailed)	0.005	0.001	0.000	0.000	0.097	0.000
number of	Corr. Coeff.	-0.056	0.088	0.008	-0.159	0.013	0.046
stigmatic rays	Sig. (2-tailed)	0.337	0.130	0.884	0.006	0.820	0.432

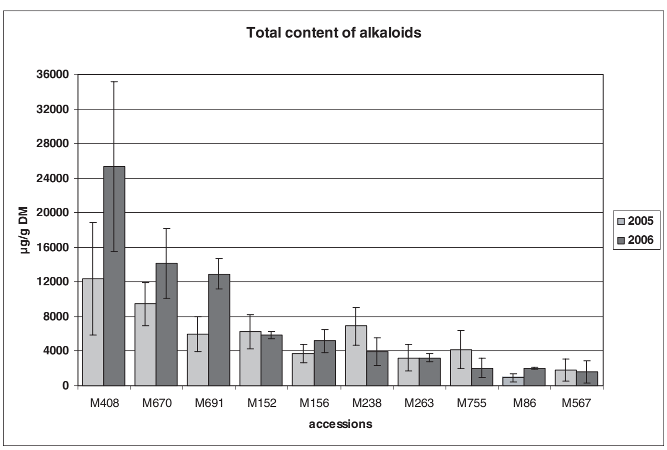

Fig. 1: Bar diagram of total content of alkaloids in 2005 and 2006 with standard deviation. (DM – dry matter)

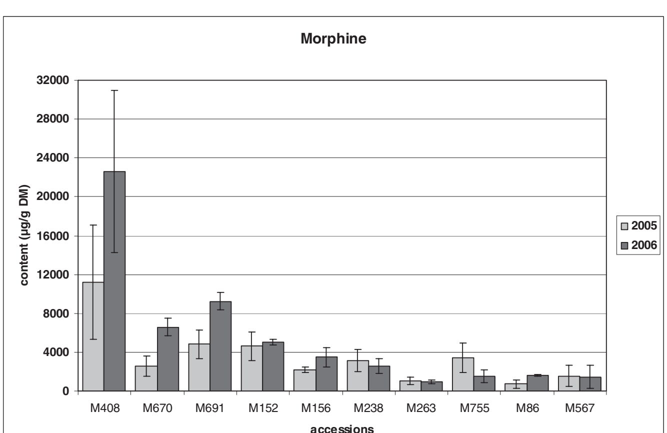

Fig. 2: Bar diagram of morphine content in 2005 and 2006 with standard deviation. (DM – dry matter)

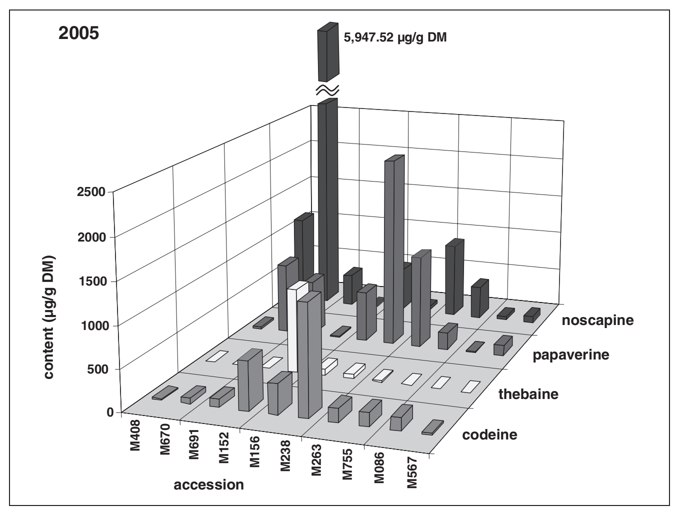

Fig. 3: Distribution pattern of codeine, thebaine, papaverine and noscapine in the year 2005.

Morphological characterisation and correlation with phytochemical data

According to the descriptor, 35 morphological and agronomic characters were collected for all 300 accessions in 2005, 2006 and 2007. Most notably, it proved difficult to distinguish between round and angular stigmatic lobes and dehiscent or indehiscent capsules in 2005 as well as 2007. In those years some accessions showed half open capsules possibly due to a long period of rain during maturity. Another problem was determining seed colour; in some cases there were different seed colours in the capsules of one plant. Determination of chromosome numbers with flow cytometry showed that the accessions of subspecies *setigerum* are tetraploid whereas all accessions of the other subspecies are diploid.

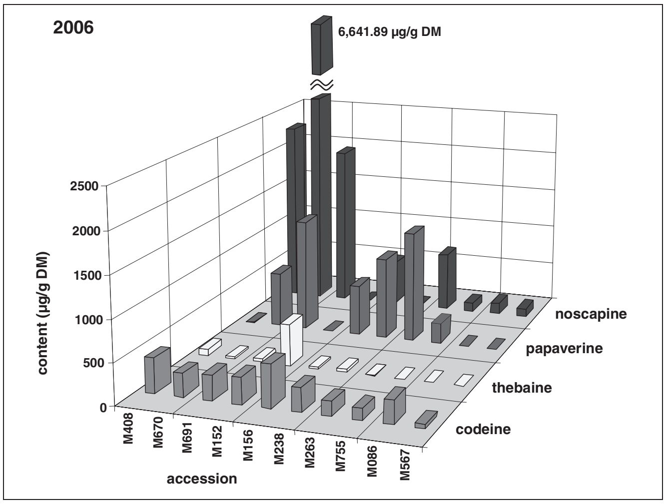

Fig. 4: Distribution pattern of codeine, thebaine, papaverine and noscapine in the year 2006. (DM – dry matter)

Only weak correlations (with correlation coefficients smaller than 0.480) were detected between any morphological characters for example plant, flower and filament colour, plant height, seed yield per capsules as well as number of stigmatic rays and the content of different alkaloids or total alkaloid content in both years. In Tab. 3, four values for 2006 are listed as an example. Furthermore, even no pattern was recognisable in the composition of alkaloids which was based on the existing infraspecific classification. The only exception was that tetraploid accessions of subsp. *setigerum* showed significant higher papaverine content than the two other subspecies.

Discussion

The qualitative and quantitative composition of the main alkaloids of all 300 accessions was highly variable, but with morphine as the main alkaloid in nearly all accessions. Shukla et al. (2006) observed within 98 germplasm lines morphine content as major constituent followed by noscapine, codeine, thebaine and papaverine. Our results showed no such clear behavior. In general, accessions had high accumulations of morphine. The position of the other main alkaloids changed comparing all accessions in all three years of cultivation. In addition, we detected several accessions with noscapine, papaverine or codeine as major constituent in all three years. Similar results are documented by Gümüşçü et al. (2008) who found two germplasm lines out of 99 which had more codeine and noscapine compared to morphine, respectively. One possible explanation of an accumulation of noscapine or papaverine could be an increased enzymatic activity in appropriate parts of the biosynthetic pathway that results in larger amounts of these alkaloids. Codeine is a precursor of morphine. Less enzymatic activity involved at a particular conversion step causing partial blocking of morphine pathway (codeine to morphine) could induce an accumulation of codeine. Tookey et al. (1976) reported about increased codeine to morphine ratio caused by lancing the capsules during maturation under controlled environmental conditions. This induced not an increase in production of codeine but rather a translocation from stem to capsules. So codeine constituent could be explained in two ways genotypic, block in alkaloid biosynthesis, or environmental. Due to the fact that codeine as major constituent was only detected within two accessions in 2005 most likely in our case is the environmental influence on its accumulation. Strong winds in 2005 could have caused damages on capsules as well as lower temperatures during capsule maturation could have influenced enzymatic activity.

During the maturation period rainfall and low temperatures might reduce alkaloid accumulation. According to Hofman and Menary (1979) three mechanisms could cause lower alkaloid content during wet weather: leaching, fungal activity and the changed activity of capsule enzymes. The amount of rainfall in the period of time from flowering until the end of maturation was higher in 2005 with 159.9 mm compared to 123.1 mm in 2006. These values were accompanied by lower temperatures in 2005. High rainfall and relative humidity permit heavy fungal growth. Their results showed that staked capsules were relatively undamaged by fungal growth compared to unstaked control capsules. Staking treatment reduced interplant abrasion and produced capsules with a comparatively intact waxy bloom (Hofman and Menary, 1984). A damaged and dewaxed capsule is more susceptible to leaching by rain. According to Hofman and Menary (1984) up to 60 and 80% of morphine and codeine content were removed from the mature capsules by leaching. In contrast, the loss of thebaine averaged only 40%. Bernáth and Tétényi (1979, 1981) demonstrated that higher temperatures as well as light intensity influenced alkaloid biosynthesis positively. The total amount of alkaloids reached a maximum value in a „high temperature“ programme under the $3.2x10^4$ lux illumination. Within this programme maximum temperatures of 26.0/16.0 °C (day/night) are used in phytotron compared to 18.5/11.5 °C (day/night) in „low temperature“ programme.

It was not possible to detect strong correlations between morphological characters like flower colour, plant height and content of major alkaloids as it was reported in Bajpai et al. (2000). They investigated 210 opium poppy lines according to morphological characters and alkaloid content. The conclusion of their work was that high morphine yield is related to large size of capsules and peduncles, small plant height, absence of pigmentation in flowers and low level of seed production. However, the mentioned correlations between morphological characters and phytochemical data showed correlation coefficients with values lower than 0.500. These data are comparable to the correlations obtained in this study in which less than 25 % of the total variance is explained by that statistical correlation. Khanna and Singh (1975) had done correlation studies in *P. somniferum* within five varieties and three strains. As one result of their work they showed that plant dry weight, capsule number and stigmatic rays are significantly correlated with opium, morphine and seed yield. Between the observed characters within our investigations for example capsule numbers per plant or stigmatic rays and content of alkaloids exist no significant correlations.

Within all 300 accessions only tetraploid subsp. *setigerum* could be separated according the hairiness of buds. Additionally, most of these lines showed a higher content of papaverine. But this alkaloid content was not distinct enough to use it as a separation character within all 300 accessions. All in all there is no important correlation between morphological characters and alkaloid content. So it is not possible to use any of these characters as a prediction tool of alkaloid content during breeding process. For this purpose it is necessary to continue measuring the alkaloid content directly.

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