Journal of Applied Botany and Food Quality 83, 14 - 18 (2009)

[1]Chair of Physiology, Centre of Life and Food Sciences (ZIEL), Technische Universitaet München, Freising-Weihenstephan
[2]Analytical Research Group, Chair of Biopolymer Chemistry, Technische Universitaet München, Freising-Weihenstephan

Effects of pine pollen extracts on the proliferation and mRNA expression of porcine ileal cell cultures

Sabine Masanetz[1], Christine Kaufmann[1], Thomas Letzel[2], Michael W. Pfaffl[1]

(Received June 6, 2009)

Summary

Masson pine pollen has been used in the traditional Chinese medicine for several hundred years and is attributed a variety of health promoting effects including effectiveness against disorders of the digestive system. But only in recent years some evidence has been found that pine pollen and its compounds do influence e.g. inflammatory activities in mice or mRNA expression profiles in piglets. At least a part of these effects have been attributed to the content of polyphenols in pollen.

In the present study different extracts of *Pinus massoniana* pollen were analyzed for their effects on cell proliferation and mRNA expression levels of selected genes. Cell proliferation was analyzed using an electronic cell impedance sensing technique and relative gene expression profiles were investigated using qRT-PCR. It was found that water and 50% ethanol extracts of Masson pine pollen at a concentration equivalent to 1% unprocessed pollen decreased cell proliferation significantly ($p<0.05$ to $p<0.01$). A 100% ethanol extract only transiently delayed cell growth ($p<0.05$ to $p<0.001$). 80% methanol and hexane extracts had no effects on cell proliferation. At the same concentration only the 50% ethanol extract led to a significant up-regulation of the relative expression levels of the pro-inflammatory genes IL-6 and IL-8 and to a down-regulation of proliferation promoter cyclin A ($p<0.05$).

LC-ESI-ToF-MS was performed to get a first impression of the compounds that may be responsible for the effects observed. Distinct mass signals have been identified that can be found in the effective pollen extracts but not in 80% methanol or hexane extracts. A further identification of additional substances could not be performed yet.

Introduction

Pollen of the Chinese yellow pine – *Pinus massoniana* – has been used in traditional Chinese medicine for a long time for its health supporting effects or to directly treat a variety of diseases including disorders of the digestive system (CHOI, 2007). In modern times pine pollen is also used in the food and cosmetic industries and its health promoting effects are advertised worldwide. But up until now only few studies have been performed to verify those claims. It has been shown that Masson pine pollen consists mostly of cell wall components delivering only little metabolisable energy (ZHAO et al., 1996). General studies of pine pollen found different carbohydrates (BOUVENG, 1963), fatty acids (SCOTT and STROHL, 1962) and finally a pattern of polyphenolic substances (STROHL and SEIKEL, 1965). Especially the latter are commonly known for their anti-oxidative, anti-carcinogenic and immuno-stimulatory effects (LAMBERT et al., 2005; SEHM et al., 2006). A more recent study found that addition of Masson pine pollen to the feed modified mRNA expression levels of inflammatory, cell cycle and growth associated genes in colon of piglets (SCHEDLE et al., 2008). CHOI (2007) reported that *Pinus densiflora* pollen extracts exhibited anti-nociceptive and anti-inflammatory activities in mice. These effects were attributed to the content of polyphenolic compounds in the pollen. Similar extracts of *Pinus densiflora* pollen were found by LEE et al. (2008) to have strong anti-oxidative and anti-inflammatory activities.

The present study was performed to investigate the effects of different extracts obtained from pollen of *Pinus massoniana* on cell proliferation and expression profiles of selected genes in a porcine ileal cell culture (IPI-21). Furthermore a first attempt was made to identify pollen compounds responsible for effects observed during this work.

Material and methods

Pollen extracts

Masson pine pollen was shock frozen with liquid nitrogen prior to breaking with a ball mill to prevent thermal damage to compounds. Subsequently 10 g of ground Masson pine pollen were extracted with 60 ml of one of the following solvents: a) water, b) 50% ethanol, c) 100% ethanol or d) a mixture of hexane and 80% methanol (v/v=1:1). Extraction was performed at room temperature for 24 h. Hexane and 80% methanol formed two distinct phases which were further processed individually. Extracts were dried using either vacuum centrifugation or lyophilization. Remaining residues were weighed and were then dissolved in phosphate buffered saline (PBS) containing 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich, Steinheim, Germany) to enhance solubility of lipophilic substances. Extracts were sterile filtered (Minisart 0.2 µm, Sartorius AG, Göttingen, Germany) and stored at -20°C. For control treatments extracts were produced with the same solvents but without addition of pollen.

Cell proliferation

Cell proliferation of the porcine ileal cell line IPI-21 was investigated with an electric cell-substrate impedance sensing device (ECIS™ Model 1600, Applied Biophysics, Troy, New York). Cells were cultured at a density of $2.0x10^4$ cells/well in 8-well arrays (ECIS Cultureware 8W10E, Applied Biophysics) fitted with 10 electrodes per well. Extracts were added to reach a final concentration equivalent to 1% (w/v) whole pollen in the medium with a total volume of 300 µl medium. Control treatments were performed with equal amounts of corresponding control extracts. Impedance was measured every three minutes at 30 kHz. Impedance results for each well were normalized to the first value obtained immediately after addition of cells and before cells were attached.

mRNA expression

After 48 h of cultivation total RNA was extracted using Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA concentrations were measured with a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) and diluted to a working solution of 10 ng/µl. RNA quality was checked to be sufficient with a Bioanalyzer 2100 (Agilent, Palo Alto, CA). qRT-PCR reactions were performed with Rotor-Gene 3000 (Corbett

Life Science, Sydney, Australia) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). Specific primer sets for all investigated genes were designed using primer3 v. 0.4.0 (http://frodo.wi.mit.edu/) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Forward and reverse primer sequences are given in Tab. 1. The run protocol consisted of reverse transcription (10 min at 50°C), denaturation (5 min at 95°C) and an amplification and quantification step consisting of 45 cycles (15 s at 95°C, 30 s at 60°C, 20 s at 68°C).

Pollen compounds

Liquid chromatography coupled by electrospray ionisation to time-of-flight mass spectrometry (LC-ESI-ToF-MS) was applied to achieve detailed information of compounds contained in the different extracts. The separation was performed with a Prontosil 120-3-C18 reversed-phase column (Bischoff Chromatography, Leonberg, Germany). After spraying the compounds via an electrospray the screening analysis was performed by simultaneous detection of positively and negatively charged ions with a high-resolution and high-accuracy time-of-flight mass spectrometer (6210 ToF LC/MS, Agilent, Santa Clara, CA). Further details can be found in a recently published study using a similar analytical set-up (GROSSE and LETZEL, 2007).

Statistical analysis

Statistical analysis of cell culture results was done with Student's t-test for each time point. Relative quantification and statistical analysis of gene expression results were performed with REST 2008 V.2.0.1 (Technische Universität München, Corbett Life Science). In both cases four controls were compared to four treated cell culture wells. A significant difference between pollen and control extract treatment was declared at values of $p<0.05$.

Results

Extraction yields

Treatment of the pollen with a ball mill resulted in nearly totally destroyed pollen structures (Fig. 1). Such good accessibility to pollen

Tab. 1: Primer pairs for quantitative RT-PCR

gene	primer sequence		RT-PCR product size
histon H3	for	ACTGGCTACAAAAGCCGCTC	232 bp
(reference)	rev	ACTTGCCTCCTGCAAAGCAC	
ubiquitin	for	AGATCCAGGATAAGGAAGGCAT	198 bp
(reference)	rev	GCTCCACCTCCAGGGTGAT	
GAPDH	for	AGCAATGCCTCCTGTACCAC	187 bp
(reference)	rev	AAGCAGGGATGATGTTCTGG	
caspase 3	for	TGTGTGCTTCTAAGCCATGG	158 bp
(pro-apoptotic)	rev	AGTTCTGTGCCTCGGCAG	
cyclin A	for	GCAGCAGCCTTTCATTTAGC	116 bp
(proliferation)	rev	TGAAGGTCCAGGAGACAAGG	
TGFβ1	for	TACTACGCCAAGGAGGTCAC	155 bp
(anti-inflammatory)	rev	TCTGCCCGAGAGAGCAATACA	
IL-6	for	AAGGTGATGCCACCTCAGAC	151 bp
(pro-inflammatory)	rev	TCTGCCAGTACCTCCTTGCT	
IL-8	for	GGCAGTTTTCCTGCTTTCTGC	153 bp
(pro-inflammatory)	rev	CAGTGGGGTCCACTCTCAAT	

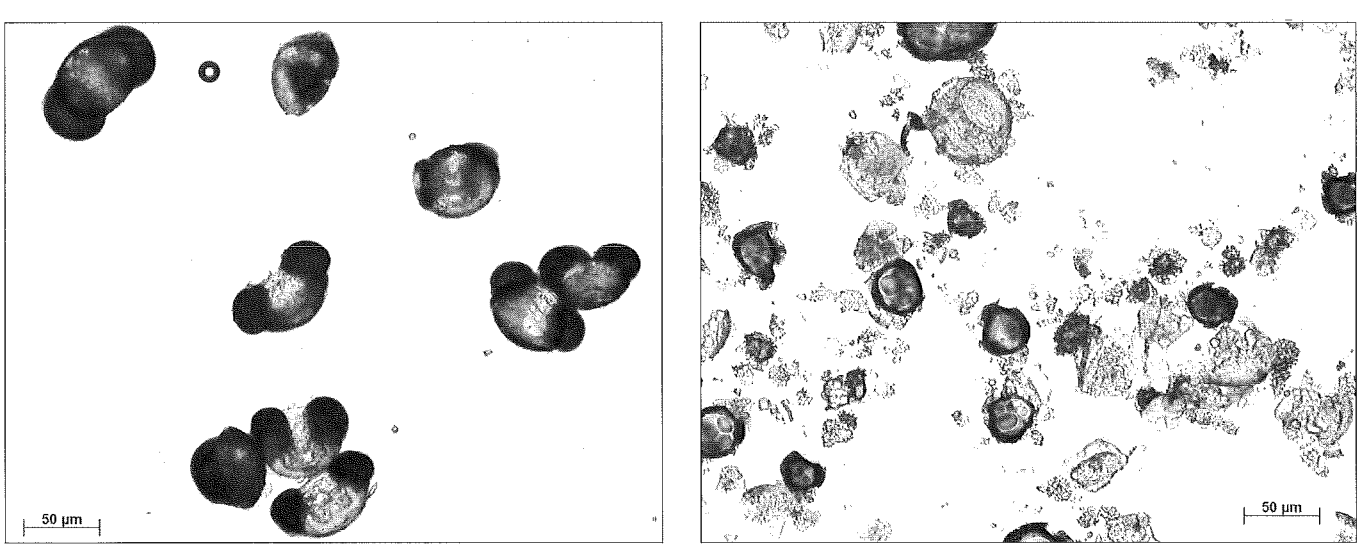

Fig. 1: Masson Pine pollen before **(A)** and after **(B)** breaking with a ball mill.

compounds for extraction should be guaranteed.
The highest extraction yields with 18.3% and 13.7% of total pollen dry matter were achieved with water and 50% ethanol respectively. 100% ethanol extracted 5.8% of pollen compounds. Hexane (6.1%) and 80% methanol (2.3%) in combination extracted 8.4% of pollen dry matter. Control extractions did not result in measurable quantities.

Cell proliferation

Investigation of cell proliferation with the ECIS device showed a high reproducibility with comparatively little variance between single growth curves within one setup. For a better graphic representation standard errors of the mean are only shown for data points every two hours.
Comparison of growth curves revealed that water (data not shown) and 50% ethanol extracts (Fig. 2) of Masson pine pollen decreased impedance values significantly to 70% ($p<0.05$) and 50% ($p<0.05$ to $p<0.01$) respectively of the values of control treated wells. Significant differences were visible after approximately 15 to 20 h after seeding. 100% ethanol pollen extract (data not shown) only transiently decreased cell densities between eight and 24 h after seeding ($p<0.05$ to $p<0.001$) to 80-90% of the control treatment. 80% methanol (data not shown) and hexane extracts (Fig. 3) had no significant effects on cell proliferation.

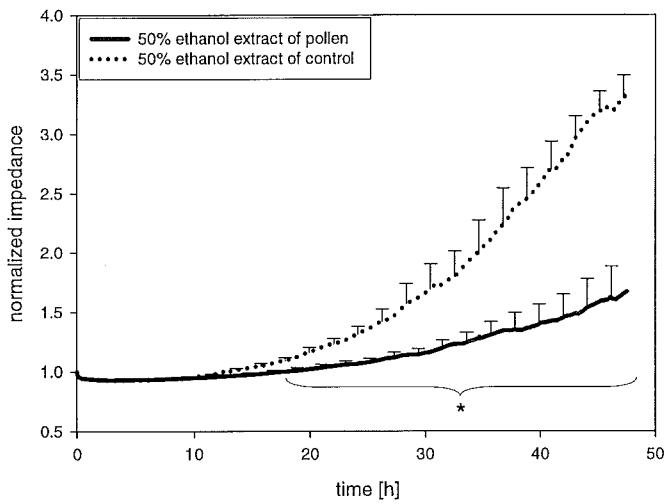

Fig. 2: Water extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium decreased normalized impedance values representing cell densities significantly from ca. 18 h after seeding when compared to cells treated with control extracts without pollen compounds. Data is represented as mean + standard error of the mean (every two hours). Asterisk shows significant differences ($p < 0.05$).

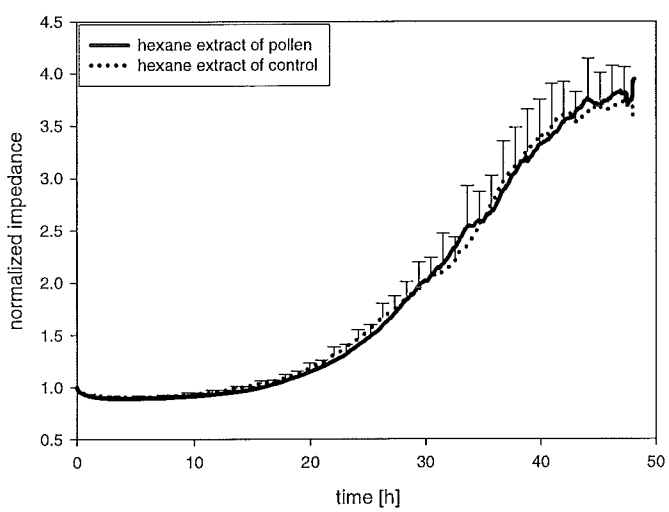

Fig. 3: Hexane extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium had no significant effects on cell growth when compared to cells treated with control extracts without pollen compounds. Data is represented as mean + standard error of the mean (every two hours).

mRNA expression

Of all pollen extracts only the 50% ethanol extract (Fig. 4) led to a significant up-regulation of mRNA expression levels of the pro-inflammatory genes IL-6 (factor 4.3, $p=0.012$) and IL-8 (factor 2.3, $p=0.024$) and to a down-regulation of the proliferation regulator cyclin A (factor 0.7, $p=0.039$). All other extracts did not have significant effects on mRNA expressions (data not shown).

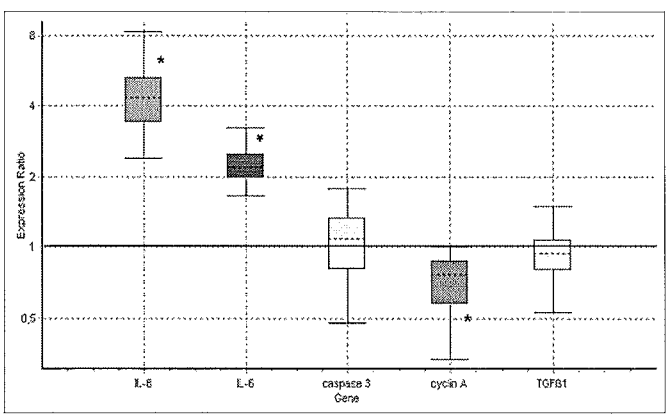

Fig. 4: 50% ethanol extract of Masson pine pollen significantly increased relative expression of IL-8 and IL-6 and decreased the relative expression of cyclin A compared to cells treated with control extracts without pollen compounds (*: $p < 0.05$)

Pollen compounds

LC-ESI-ToF-MS was performed in order to characterize the compounds responsible for the effects observed. Thereby a number of distinct mass signals has been identified that can be found exclusively in the three pollen extracts that exhibited effects on cell proliferation and/or gene expression (Tab. 2). These signals were not detected in both 80% methanol and hexane extracts of Masson pine pollen. A detailed identification of these substances was not yet possible due to missing reference substances. However, most compounds could be characterized via hydrophobicity (retention factor), ionization behavior and the molecular formula. A database containing the three independent parameters (BERKEMEYER and LETZEL, 2008) will identify these compounds very specific in forthcoming samples.

Discussion

Traditional Chinese medicine and its products are a growing market in the western countries. Masson pine pollen is one of these products heavily advertised in internet stores for its health promoting effects. The present study aimed to verify some of these claims. Therefore

Tab. 2: LC-ESI-MS signals specific for effective Masson pine pollen extracts.

	water extract	50% ethanol extract	100% ethanol extract
m/z (-)		135.050	
	146.107	146.107	
	204.094	204.094	
	230.167		
	328.231	328.230	
	330.235		330.199
m/z (+)		129.086	
	131.101		
	135.053		
	146.104		
	235.126	235.126	235.111
		237.097	
	254.127		
		318.217	
		334.218	

a combination of a real-time cell monitoring system, qRT-PCR and LC-ESI-ToF-MS was used for the first time.
Especially the ECIS system enables to monitor changes in impedance values of a cell culture at very short intervals with a high reproducibility. Such it was possible to compare cell growth of four repetitions of each pollen extract and mock treated culture wells with 960 single t-tests – every three minutes for 48 h. A resolution that was not possible with conventional cell culture techniques.

The yields of the extraction methods (up to 18.3% w/w for the water extract) used in this study were relatively similar to the yield obtained by CHOI (2007). Their extraction of intact *Pinus densiflora* pollen with 70% ethanol for three days at room temperature resulted in a yield of 8%. But since in the present study pollen was broken prior to extraction it is to be expected that a broader range of pollen compounds is reached simultaneously.

Since most of the research work on pollen effects is done in feeding experiments there is only little data available on pollen effects on cell growth and gene expression *in vitro*. In a piglet feeding study addition of *Pinus massoniana* pollen resulted in increased villus heights in the small intestine (SCHEDLE et al., 2008). In feeding experiments effects of pine pollen are often attributed to the increase of ingestion of cell wall components (ZHAO et al., 1996). Since non-soluble cell wall constituents should be absent in the pollen extracts it is likely that other pollen compounds trigger the results of the present study. Additionally a decrease in cell proliferation is rather opposed to the increase of villus lengths observed in the piglet study.
The bioactive properties of bee pollen extracts (KROYER and HEGEDUS, 2001) as well as the anti-nociceptive and anti-inflammatory properties of extracts from *Pinus densiflora* pollen (CHOI, 2007) have been attributed to the high content of polyphenolic substances. Since polyphenols of different plants have been found to decrease cell proliferation in different cell lines (e.g. KANEUCHI et al., 2003; BRIVIBA et al., 2002; IIJIMA et al., 2000) such compounds of the Masson pine pollen could be an explanation for the results observed in the present study. Different types of polyphenols also may show different solubilities in lipophilic and hydrophilic solvents so it is possible that not all extracts lead to similar decreases of cell proliferation.
Water and 50% ethanol extracts revealed similar effects on cell proliferation. But only substances in the 50% ethanol extract appeared to influence mRNA expression of cyclin A as a possible explanation for the decrease of proliferation observed. It is to be expected that additional regulators of cell proliferation or cell death are also targeted by water soluble substances that may also be present in the 50% ethanol extract. A similar regulation of cyclin A has been found before for red wine polyphenols (IIJIMA et al., 2000).
A modulation of the pro-inflammatory IL-6 and IL-8 suggests that pollen compounds present in the 50% ethanol extract are able to alter the fine tuning of the inflammatory status of ileal cells. The regulations found in the present study hint to an increase of the inflammatory status of the cultured cells. In contrast to this polyphenols mostly have been found to have anti-inflammatory properties. Nevertheless there is evidence for some polyphenolic substances – e.g. *cis*-resveratrol – to increase expression of pro-inflammatory interleukins such as IL-6 (LEIRO et al., 2005). But in contrary to the findings of the present study 70% ethanol extracts of *Pinus densiflora* pollen have been found to inhibit the production of pro-inflammatory signal molecules – including IL-6 – in macrophages after activation with LPS (LEE et al., 2009).
Unfortunately none of the pollen compounds could be identified with the methods available for the present study. Nevertheless it was possible to at least identify mass signals of compounds exclusively found in the water, 50% ethanol and 100% ethanol extracts. Such one or a combination of some of these may be responsible for the effects observed with those extracts.

References

BERKEMEYER, C., LETZEL, T., 2008: LC-API-MS(/MS) for biological samples: Importance of hydrophobicity, molecular weight and structural development. LCGC Europe 21, 548-561.

BOUVENG, H.O., 1963: Polysaccharides in pollen. I. Investigation of mountain pine (*Pinus mugo* turra) pollen. Phytochemistry 2, 341-352.

BRIVIBA, K., PAN, L., RECHKEMMER, G., 2002: Red wine polyphenols inhibit the growth of colon carcinoma cells and modulate the activation pattern of mitogen-activated protein kinases. J. Nutr. 132, 2814-2818.

CHOI, E., 2007: Antinociceptive and antiinflammatory activities of pine (*Pinus densiflora*) pollen extract. Phytotherapy Res. 21, 471-475.

GROSSE, S., LETZEL, T., 2007: Liquid chromatography/atmospheric pressure ionization mass spectrometry with post-column liquid mixing for the efficient determination of partially oxidized polycyclic aromatic hydrocarbons. J. Chromatogr. A 1139, 75-83.

IIJIMA, K., YOSHIZUMI, M., HASHIMOTO, M., KIM, S., ETO, M., AKO, J., LIANG, Y., SUDOH, N., HOSODA, K., NAKAHARA, K., TOBA, K., OUCHI, Y., 2000: Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin A gene. Circulation 101, 805-811.

KANEUCHI, M., SASAKI, M., TANAKA, Y., SAKURAGI, N., FUJIMOTO, S., DAHIYA, R., 2003: Quercetin regulates growth of Ishikawa cells through the suppression of EGF and cyclin D1. Int. J. Oncol. 22, 159-164.

KROYER, G., HEGEDUS, N., 2001: Evaluation of bioactive properties of pollen extracts as functional dietary food supplement. Innov. Food Sci. Emerg. Technol. 2, 171-174.

LAMBERT, J.D., HONG, J., YANG, G., LIAO, J., YANG, C.S., 2005: Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am. J. Clin. Nutr. 81, 284S-291S.

LEE, K., KIM, A., CHOI, E., 2009: Antioxidant and antiinflammatory activity of pine pollen extract *in vitro*. Phytotherapy Res. 23, 41-48.

LEIRO, J., ARRANZ, J.A., FRAIZ, N., SANMARTIN, M.L., QUEZEDA, E., ORALLO, F., 2005: Effect of *cis*-resveratrol on genes involved in nuclear factor kappa B signaling. Int. Immunopharmacol. 5, 393-406.

SCHEDLE, K., PFAFFL, M.W., PLITZNER, C., MEYER, H.H.D., WINDISCH, W., 2008: Effect of insoluble fibre on intestinal morphology and mRNA expression pattern of inflammatory, cell cycle and growth marker genes in a piglet model. Arch. Anim. Nutr. 62, 427-438.

SCOTT, R.W., STROHL, M.J., 1962: Extraction and identification of lipids from loblolly pine pollen. Phytochemistry 1, 189-193.

SEHM, J., LINDERMAYER, H., MEYER, H.H.D., PFAFFL, M.W., 2006: The influence of apple- and red-wine pomace rich diet on mRNA expression of inflammatory and apoptotic markers in different piglet organs. Anim. Sci. 82, 877-887.

STROHL, M.J., SEIKEL, M.K., 1965: Polyphenols of pine pollens. Phytochemistry 4, 383-399.

ZHAO, L., WINDISCH, W., KIRCHGESSNER, M., 1996: A study on the nutritive value of pollen from the Chinese Masson Pine (*Pinus massoniana*) and its effect on fecal characteristics in rats. Z. Ernähr. Wiss. 35, 341-347.

Address of the authors:
Sabine Masanetz, M.Sc., ZIEL – Lehrstuhl für Physiologie, Weihenstephaner Berg 3, 85354 Freising, Germany, masanetz@wzw.tum.de
Christine Kaufmann, ZIEL – Lehrstuhl für Physiologie, Weihenstephaner Berg 3, 85354 Freising, Germany
Dr. Thomas Letzel, Competence Pool Weihenstephan (CPW), Weihenstephaner Steig 23, 85354 Freising, Germany
Dr. Michael W. Pfaffl, ZIEL – Lehrstuhl für Physiologie, Weihenstephaner Berg 3, 85354 Freising, Germany