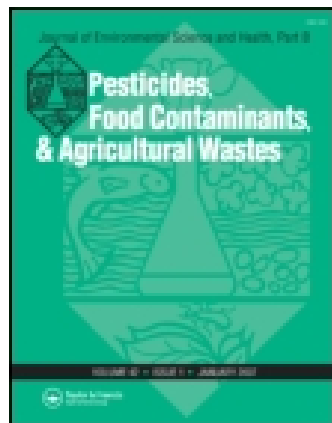




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Variability of *Corylus Avellana*, L. CorA and profilin pollen allergens expression

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Corylus avellana is the source of inhalant allergies induced by hazel pollen as well as food allergies induced after ingestion of hazelnuts. In this study, real-time PCR approach was used to analyse expression of hazel pollen allergens on the molecular level. Relative quantity of hazelnut allergens *Corylus avellana*, L. CorA and *Corylus avellana*, L. pollen profiling in samples from different Ukraine areas were determining and comparing. Differences among the levels of both analysed allergen transcripts were found for hazel CorA and profilin. In both cases, the expression within the urbanized growth conditions was higher when compared to the sample from village area. The average expression for CorA was 0.84 times higher than for profilin and the results are very variable depending on the place of growth. Expression levels here were within the range of 2.957 up to the 52.936. Profilin expression was the highest in the sample from the polluted place of growth–cement plant area with the value of 52 times higher when compared to the sample from the village area. In this study, comparison of expression levels of hazel CorA and profiling pollen allergens was performed for the first time. Real-time PCR assay developed in this study proved the sensitivity for detection of the changes of the hazel pollen allergens expression levels and could benefit labs by fast and reproducible detection method of these allergens.

Keywords: *Corylus avellana*, pollen allergens, CorA allergen, profilin allergen, gene expression.

Introduction

Pollen allergens are one of the major sources of respiratory diseases.^[1,2] Allergens as generally airborne particles represented by pollen spores caused many of allergic symptoms, when eyes, nose and lungs are exposed to these particles.^[3] In urbanized area throughout the world, an increasing tendency is reported for the prevalence of allergic mainly allergic respiratory diseases. D'Amato et al.^[4] explained this by changes in environmental factors, including indoor and outdoor air pollution. Hypersensitivity to aeroallergens causes allergic diseases such as allergic rhinitis, irritation or running of the nose, sneezing, itching and redness of the eyes that affect up to 30% of the population.^[5,6] Allergens when inhaled also cause asthmatic symptoms like bronchoconstriction because it increases the production of mucus in the lungs along with dyspnea, coughing and wheezing which are common symptoms of

the disease.^[3] Therefore, questions about allergy and sources of allergens are often discussed and studied.^[7,8]

Plants infected by pathogens, injured or attacked by exogenous stimuli, induce a series of defence responses to protect themselves that lead to the producing of defence-related proteins.^[9] By attaching to the surface of pollen grains, pollutants could modify not only the morphology of these antigen-carrying agents but also their allergenic potential. In addition, by inducing airway inflammation, which increases airway permeability, pollutants overcome the mucosal barrier and could be able to “prime” allergen-induced responses.^[4]

Hazel (*Corylus avellana*) is known to cause inhalant allergies induced by sensitization with hazel pollen as well as provoking food allergy after its ingestion.^[10] In some study,^[10] authors also summarized that normally, hazelnut allergy is associated with tree pollen allergy. For major pollen allergens of trees from the order *Fagales* – especially alder, birch, hazel and hornbeam – an immunological cross-reactivity has been reported in studies before. Evidence for cross-reactivity between birch pollen allergens and those from vegetables, fruits and nuts was obtained by identifying allergen structures that share similar IgE binding sites.^[11–13]

In hazel, two pollen allergens were described by Hirschehr et al.^[14] Authors of the study identified major

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allergens in hazelnut (18 kDa) and hazel pollen (17 kDa) related to the birch pollen allergen BetV1 as well as the minor 14 kDa hazelnut allergen related to birch profiling BetV2.^[15]

In the reported study from Ukraine, hazelnut pollen was analysed for the expression of two above-mentioned allergens. Savitsky et al.^[16] presented the results of aeropalynological observations in Kyiv, carried out with a gravimetric method, during January–October, 1994. The six most abundant pollen types were: *Betulaceae* (21%), *Chenopodiaceae/Amaranthaceae* (10%), *Ambrosia* (10%), *Artemisia* (9%), *Pinaceae* (8%) and *Poaceae* (6%).

Real-time PCR approach was used for the analyses of allergens on the molecular level. This tool for evaluating of birch pollen allergens was used before by Žiarovská et al.^[17] Longhi et al.^[18] used real-time PCR as a rapid, accurate, and automated tool for the detection and quantification of airborne allergenic pollen taxa. Real-time PCR is also a useful tool for detection: gene expression^[19] identification and quantification of pathogens or allergens,^[20–22] and authentication of food.^[23,24]

The aim of the study was to determinate and compare relative quantity of hazelnut allergens *Corylus avellana*, *L. CorA* and *Corylus avellana*, *L.* pollen profiling in pollen samples from different Ukraine areas by real-time PCR.

Materials and methods

Pollen samples and RNA preparation protocol

Expression level of *CorA* and profilin genes of *Corylus avellana*, *L.* were analyzed by using of the quantitative real-time PCR. Hazelnut pollen was sampled in Ukraine areas as listed in Table 1. Pollen of *Corylus avellana*, *L.* has been prepared prior to the beginning of anthesis in Kyiv, Kamenets-Podolskiy and Sumy regions of Ukraine. Pollen for analysis was sampled after flowering time, soon after anther dehiscence. Samples were desiccated and freeze-dried in plastic aseptic container until further analysis.

Total RNA from all analysed pollen samples was extracted using the GeneJETTM Plant RNA Purification Mini Kit (Fermentas, Slovak Republic) according to

the manufacturer's recommendations and RNA quality and the concentration were quantified by spectrophotometer. Reverse transcription was performed with the RevertAidTM First Strand cDNA Synthesis Kit (ThermoScientific, Slovak Republic) using 40 ng of total RNA extracted from pollen, according to the manufacturer's recommendations and with temperature and time profile listed in the manufacturer protocol.

Real-time PCR analysis and data processing

Primers for real-time PCR were developed on the basis of the mRNA sequences *CorA* and profilin genes. Conserved parts of *CorA* mRNA accessions from The National Centre of Biotechnology Information database: NCBI (DQ663552, DQ663551, DQ663550, DQ663549, DQ663548, DQ663547, DQ663546, DQ663545, DQ663544 and DQ663543) and conserved parts of profilin mRNA accessions (X71000, X70999, X70998 and X70997) were used for primer designing. Nucleotide sequence for 18S rRNA (NCBI accession AY263895) was used as housekeeping gene for data normalization. Primers were designed using Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>; Bethesda, MD, USA). Primer sequences as used for real-time PCR assays are listed in Table 2.

To optimize the PCR conditions for each primer, annealing temperature and PCR efficiency by the standard curve were performed. The specificity of the PCR amplification was checked by a melt curve analysis and cross-confirmed by agarose gel electrophoresis.

All reactions were performed in a Biorad (Slovak Republic) CFX96 machine. Primer concentrations were independently optimized to favour product formation then amplification efficiency for each optimized primer pair was calculated using a 10-fold dilution series. Real-time PCR reactions were performed in the volume of 25 µL containing 2xSensiMixTM SYBR No-ROX Master mix (ThermoScientific), 0.25 µM of each primer and 135 ng of transcribed cDNA. The temperature and time conditions were as follows: initial denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s, 58°C for 15 s and 72°C for 15 s. Fluorescence was read at 58°C during each cycle. Melting curves were subsequently determined for each reaction to ensure that single products were produced and the resulting reaction was run on a 1.8%

Table 1. Pollen samples of *Corylus avellana*, *L.* Ukraine.

Sample	Place of growth	Note
K1	Kyiv – M.M.Gryshko National Botanic Garden	City
K2	Kyiv – V.A. Fomin National Botanic Garden; downtown near the subway	City
K3	Kyiv – garden, near downtown area	City
KP1	Kamenets-Podolskiy – Botanical Garden	City
KP2	Kamenets-Podolskiy – near the cement plant area	City
S	Sumy region	Village

Table 2. The sequences of primers used in this study.

Name of primer	Sequences of primer	Melting point of primer
CorAv_a-F	5' acaagtacgtgaaggagagg 3'	57.4°C
CorAv_a-R	5' aggtctcaaccgccctaaa 3'	55.4°C
CorAv_prof-F	5' ggagctgtcatccgtggaaa 3'	57.5°C
CorAv_prof-R	5' atgttgactgtcctgggt 3'	55.4°C
Cor18S-F	5' ctggtcttaattggccgggt 3'	57.5°C
Cor18S-R	5' aggccaacacaataggaccg 3'	57.5°C

agarose gel to confirm that amplified product was unique and of the correct size. Triplicate preps were subjected to qRT-PCR. Hazel CorA and profilin allergen quantitation was performed according to the Pfaffl method.^[25] Allergen qRT-PCR results were normalised against the 18S rRNA gene and compared to the expression of the same allergen gene in pollen sample 6 (Sumy region) that was chosen as the calibrator.

Results and discussion

In the present study, we aimed to develop a real-time PCR method for identifying and quantifying a subset of the CorA and profilin allergens of *Corylus avellana* pollen. Until now, only a real-time PCR method based on the detection of random amplified polymorphic DNA-derived sequence was reported for identification of hazelnut allergenic pollen.^[18] Another situation is within the detection of hazelnut food allergy, where a real-time PCR approach is a well established method.^[26,27] Similar approaches based on real-time PCR have already been applied in many different fields to the detection and quantification from transgene constructs^[28] to the allergens contained in foods.^[29,30]

Bioinformatic evaluation of nucleotide data for mRNAs of hazelnuts CorA and profilin pollen allergens

Firstly, *in silico* analysis was performed to identify conserved parts of both analysed allergens, as more accessions exist in database for both of them. NCBI database was queried for all existing sequences of mRNAs of hazelnuts CorA and profilin allergens. Performing a BLAST analysis^[31] against the individual sequences of both analysed allergens themselves, *in silico* conserved regions were identified. When comparing all other existing sequences of CorA to the CorA I/11 mRNA (X70997), the sequences from 61 to the 120 nt; from 181 to the 240 nt and from 426 to the 480 nt of CorA I/11 were found as to be conserved in all accessions. By comparison of all other existing sequences of *Corylus avellana* pollen profiling variant 1 mRNA (DQ663543), the sequences from 61 up to the 360 were found as to be conserved in all accessions. Based on

obtained bioinformatics data, conserved regions were used for primer designing.

Standard curve preparation and normalization of real-time PCR Assay

Standard curves were realized by analysing 5 serial dilutions of pollen cDNA in 10-fold dilutions achieving the efficiency of 97.2% for CorA allergen, 95.6% for profilin allergen and 95.2% for 18S rRNA. Standard curves showed a linear regression between input cDNA and Ct values in the two independent assays, with determination coefficients (R^2) of 0.97 and 0.99 for CorA allergen and 0.96 and 0.99 for profilin allergen. The standard curve realized with pollen samples from different places of growth showed good reproducibility, too, because the linear regression analyses demonstrated well-overlapping for the slope values. For the correction of an existing sample-to-sample variation, normalization against the control gene was used for both, control gene and calibrator served as positive controls. 18S rRNA was used as housekeeping gene for normalization. Using of 18S rRNA as internal control in gene expression analysis is reported for many plant species, such as chickpea, rice, agave or potato.^[32,33,34] For agave, 18S rRNA was concluded as good internal control,^[34] because it showed stable expression and no effect of experimental conditions.

Relative expression of hazel CorA allergen

Hazel CorA allergen was characterized as the major allergen by Breiteneder et al.^[35] and its comparison to the BetV1 or birch pollen in general was performed in many studies.^[36,37] Nowadays, pollen allergens from hazel, birch and alder are connected not only direct by airborne allergies, but they seem to be responsible through the hazelnuts for so called birch pollen-hazelnut allergy syndrome, too.^[14,38]

End-point PCR analyses revealed that the CorA allergen was transcribed in all hazel pollen samples (Fig. 1). The expression of CorA allergen gene was analysed upon various growth conditions in Ukraine (Table 1). Sample from a village growth conditions was chosen as a calibrator for expression analyses. qRT-PCR analyses confirmed

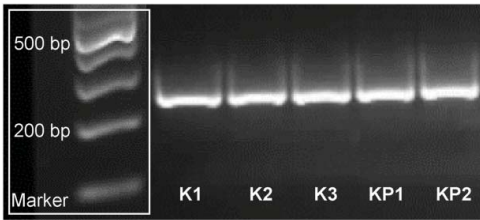


Fig. 1. Checking of specificity for CorA allergen sequences amplification in analysed pollen samples. Codes of samples as listed in Table 1.

the results of the end-point PCR, and enabled a more reliable quantification of CorA gene expression levels. qRT-PCR showed only a little variation in the abundance of allergen transcripts among the samples from different places of growth (Fig. 2). Expression levels of CorA allergen were within the range of 0.532 up to the 1.206 higher when comparing to the calibrator (pollen from Sumy region). As the sample from village served as the calibrator, the findings are very similar to those for birch pollen allergen.^[17] In our previous study, in samples from urbanized area was the expression of Betv1 allergen in average 1.5 times higher when comparing to the forest sample served as a calibrator. In samples from borders of the urbanized area was the expression of Betv1 allergen only 0.55 times higher when comparing to the forest sample.^[17] Bryce et al.^[39] also reported that extracts from pollen collected within the trees in urban areas had higher chemotactic activity on human neutrophils compared to pollen from rural sites, although the allergen content remained unchanged.

Relative expression of hazel profilin allergen

Jimenez-Lopez et al.^[38] summarized, that up to date, over 400 profilin sequences from 100 plant species are available at NCBI GenBank databases. Among these, about half

have been isolated from pollen, mostly from allergenic plant species including the allergens Ole e 2, Bet v 2, Cor a 2, Phl p 12 and Zea m 12. In the same study, authors analysed the ranges of identity percentage calculated for both the nucleotide and the amino acid sequences of profilins of following species: *Olea europaea*, *Corylus avellana*, *Betula pendula*, *Phleum pratense* and *Zea mays*. Intraspecific profilin identity range from 75.7 up to the 100% for nucleotide sequences and from 77.4 up to the 100% for amino acid ones. Interspecific profiling identity among *Betulaceae* as an important allergy source in Europe is reported^[38] as ranged from 74.3 up to the 100% for nucleotide sequences and from 77.8 up to the 100% for amino acid sequences.

In our study, qRT-PCR showed only a high variation in the abundance of profilin allergen transcripts among the samples from different places of growth. As the case of CorA, the specificity of product amplification, melt point analysis was performed (Fig. 3). Obtained values of melting point of amplicons were set as 79°C for profilin and 83.5°C for CorA allergen. Expression levels of profilin allergen (Fig. 4) were within the range of 2.957 up to the 52.936 higher when comparing to the calibrator (pollen from Sumy region). For both samples from Botanical Gardens localized in the cities as the places of growth (samples K1 and K-P1), the difference in the profilin expression was the lowest, but still higher (2.957 and 4.076 times) when comparing to the calibrator. Samples from downtown area reached the profilin expression in average 21 times higher. Profilin expression was the highest in the sample from the most polluted place of growth (cement plant area) with the value of 52 times higher when compared to the calibrator.

Comparison of levels of expression of analysed allergens in urbanized area

Increased morbidity and mortality due to the allergic disorders is of major global concern today. About 30% of the

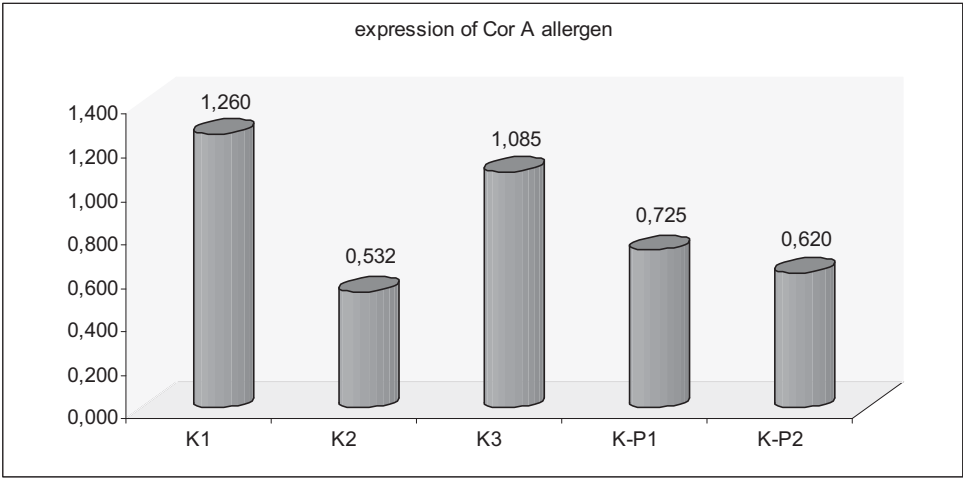


Fig. 2. Visualization of the expression ratios of CorA allergen for analysed pollen samples. Codes of samples as in Table 1.

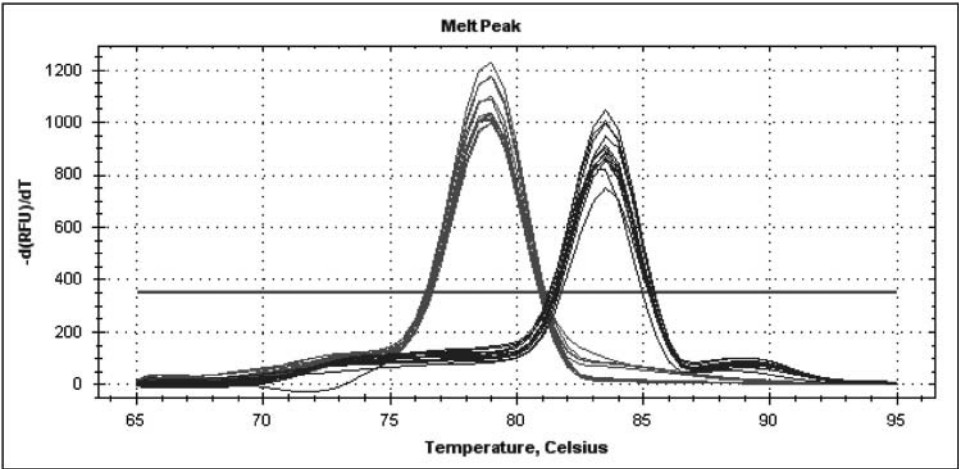


Fig. 3. Melting temperatures of analyzed hazel allergens profilin and CorA (from right to left) as visualized by dissociation curves.

global population is estimated to suffer from some of allergic diseases. Among biopollutants responsible for precipitating the allergic symptoms in susceptible individuals, pollen are the major baeroallergen and it has been observed that wide variety of pollen are responsible for eliciting the allergic symptoms in different parts of the world. [3] Differences among levels of expression are well documented for many allergens within different stages of the growth,[40,41] and studies about different expression levels of allergen for variable climatic conditions exist.[6]

In this study the comparison of expression level of hazel CorA and profilin allergen was proved for five different pollen samples that were collected in urbanized area of Ukraine. Real-time PCR based measurement of the expression level of hazel pollen allergen we have developed is in agreement with published data proposing qRT-PCR as a reliable and relatively simple method to infer allergen abundance.[40] Differences among the levels of both analysed allergen transcripts were found for hazel CorA and profilin. In both cases, the expression in the urbanized growth conditions was higher when compared to the

sample from village area, but the average expression for CorA was 0.84 times higher than for profilin. The results are very variable depending on the place of growth, as summarized below.

As hazel pollen allergens were described [14] related to the birch pollen allergen Bet v 1 and birch profiling Bet v 2, discussing of some parallels with their expression in another reported species can be performed. Bet v 1 allergen expression study was realized [17] for birch pollen from Ukraine. In samples from urbanized area was the expression of BetV1 allergen in average 1.5 times higher when comparing to the forest sample served as a calibrator. In samples from borders of the urbanized area was the expression of BetV1 allergen only 0.55 times higher when comparing to the forest sample. The expression levels of hazel CorA allergen as an analog for Bet v 1 reported here were obtained with very similar values.

Rodríguez-Perez et al.[42] reported a closed relationship for members of the profiling family from fruits, seeds, vegetables and pollens. For birch pollen profilin is also reported the high level of expression when used it as

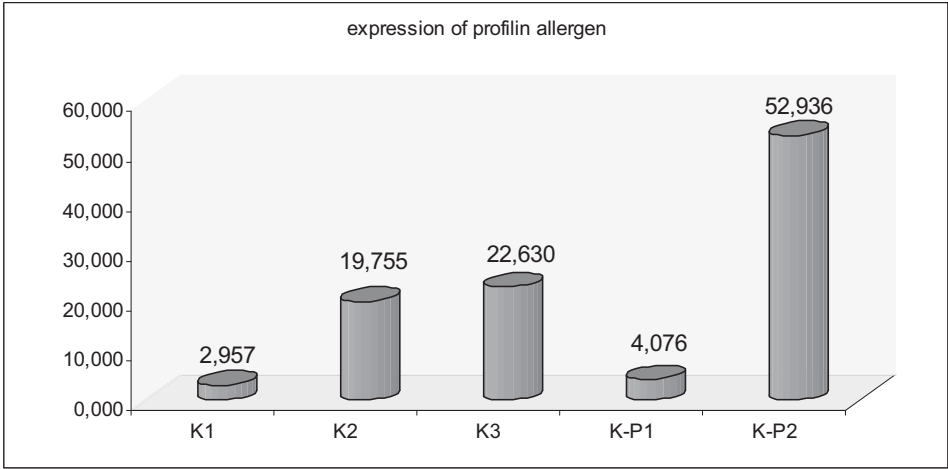


Fig. 4. Visualization of the expression ratios of profilin allergen for analysed pollen samples. Codes of samples as in Table 1.

recombinant allergen in *Escherichia coli*.^[43] As this approach is not the same as qPCR based analyse, much more higher level of profilin expression was obtained within the same samples when comparing to the CorA in this study, too.

Findings reported here and in the study of Žiarovská et al.^[17] are not only comparable but fully in concordance the findings of Bryce et al.^[39] who reported that extracts from birch pollen collected in urban areas had higher chemotactic activity on human neutrophils compared to pollen from rural sites, although the allergen content remained unchanged. Exacerbating potential of abundant airborne pollutants in urban areas for allergic reaction was reported,^[44] too. Nitrogen dioxide exposure is reported^[45] as adversely affecting the pollen germination of hazel, birch and alder, changing their protein content, including allergens. This can partial be an explanation, why studies on pollen allergen^[44,46] revealed a discrepancy between the numbers of pollen grains and quantities of pollen allergen in the atmosphere. D'Amato et al.^[45,47] further reported, that substantial airborne pollen-allergenic activity has been found, not only after, but even before, the period of the presence of pollen grains. In this study, based on the found differences among hazel pollen allergens expression, a power of qPCR for revealing of allergenic potential of pollen from different localities has been proved.

Conclusion

Urbanization is one of the aspects that correlated with the increasing number of pollen-induced respiratory allergy. Here, comparison of hazel CorA and profiling pollen allergens was performed for the first time and real-time PCR protocol for their expression was developed. q-PCR assays based on SYBR Green were targeting hazel pollen allergens and applied for *Corylus avellana* pollen samples from different regions of Ukraine. Results indicate that q-PCR assay developed in this study could sensitively detect the changes of the expression levels of the hazel pollen allergens and could benefit labs by fast and reproducible method of these allergens detection.

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