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Contribution to pollen automatic identification and assessment of atmospheric pollutants effects on pollen grains

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Abstract

Pollen identification and quantification is used in many fields of application and research. Research has been conducted to attain accurate automatic pollen recognition, aiming reducing the laborious work and subjectivity in human identification. One of the aims of this study was to evaluate the capacity of the Raman parameters of pollen spectra to be used as a future technique in pollen automatic identification. These parameters were calculated for only seven common band intervals in a limited spectral range, which were chosen to be used in the classification of the pollen species using Support Vector Machine.

The results obtained showed it is possible, using only Raman parameters of 7 peaks common to all spectra, to obtain a 100% correct classification in the training step of the classification algorithm, and 90% in the validation step. These results support the use of this methodology for the automatic identification of pollen.

The concern with air quality has been rising, and measures by the European Union for the reduction of pollutant emissions caused some improvement, especially with the reduction of the concentrations of some of the most impactful atmospheric pollutants, like the NO_X and particulate matter.

Even so, and especially in urbanized areas, the concentrations of these pollutants are high and can be harmful to human health. However, we cannot consider only the direct effects, it is extremely important to also consider the effects that pollutants cause on the environment around us. Over the last few years, many studies have been carried out to understand the influence of air pollutants on the pollen grain and what it can mean to human health and the survival of plant species.

Many studies are focused on the influence of pollutants on pollen, but few compare different pollen species subject to the same experimental conditions. In this work the changes occurring are studied, which are not the same for the 4 pollen species. In general, the NO_2 pollutant gas seems to affect all species greater than O_3, in the different parameters studied: viability, oxidative stress and soluble protein percentage. For O_3, it was possible to observe that the greater effect happened for pollen exposed to the limit concentration, which might mean that for some species the limit of tolerance is reached at this level of concentration.

Keywords: pollen; identification; pollution; health; allergenicity

Resumo

A identificação e quantificação do pólen é usada em muitos campos de aplicação e investigação. Muitos estudos têm sido conduzidos para obter a identificação automática e precisa do pólen, visando reduzir o trabalho extenso e a subjetividade na identificação humana. Um dos objetivos deste estudo foi avaliar a capacidade dos parâmetros Raman de espectros polínicos, para serem utilizados como futura técnica de identificação automática de pólen. Estes parâmetros foram calculados para apenas sete intervalos de bandas comuns em uma faixa espectral limitada, escolhidos para serem utilizados na classificação das espécies de pólen usando Machine Learning.

Os resultados obtidos mostraram ser possível, com base nos parâmetros Raman de 7 picos comuns a todos os espetros, obter 100% de correta classificação na fase de treino do algoritmo, e 90% na fase de validação. Estes resultados corroboram a utilização desta metodologia no reconhecimento automático de pólen.

A preocupação com a qualidade do ar tem vindo a aumentar, e medidas impostas pela União Europeia de redução da emissão de poluentes permitiram melhorias, principalmente na redução da concentração de alguns dos poluentes atmosféricos mais impactantes, como os NO_X e o material particulado.

Mesmo assim, as concentrações desses poluentes são altas e podem ser prejudiciais à saúde humana. No entanto, é extremamente importante considerar também os efeitos que os poluentes causam nos ecossistemas que indiretamente contribuem para o desenvolvimento da sociedade. O nosso estudo teve também como objetivo contribuir para entender a influência dos poluentes atmosféricos nos grãos de pólen e o que isso pode significar para a sobrevivência das espécies vegetais.

Muitos estudos são focados na influência dos poluentes no pólen, mas poucos comparam diferentes espécies polínicas sujeitas a diferentes poluentes sob as mesmas condições experimentais. Neste trabalho são estudadas as alterações que ocorrem, que não são iguais nas 4 espécies polínicas. No geral, o gás poluente NO_2 parece afetar todas as espécies mais do que o O_3, nos diferentes parâmetros avaliados: viabilidade, stress oxidativo e teor de proteínas solúveis. Para o O_3, foi possível observar que o maior efeito ocorreu para o pólen exposto ao valor limite de concentração, o que pode significar que para algumas espécies o limiar de tolerância ocorre neste nível de concentração.

Palavras-chave: pólen; identificação; poluição; saúde; alergenicidade.

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List of abbreviations

DNA – Deoxyribonucleic Acid

UV-LIF – Ultraviolet Laser-Induced Fluorescence

FTIR – Fourier-Transform Infrared Spectroscopy

CA – Classification Accuracy

FWHM - Full Width at Half Maximum

SVM – Support Vector Machine

PMs – Particulate Matters

ROS – Reactive Oxygen Species

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

SOD enzyme – Superoxide Dismutase enzyme

TSP – Total Soluble Protein

Dissertation outline

The core of this Dissertation is based on two peer-reviewed Articles complemented with the sections Introduction, state of the art, general discussion and conclusion presented as follows:

Chapter 1 - Introduction - in this section we present the main problem studied, emphasizing the importance of airborne pollen monitoring, the challenges of pollen identification and the influence of atmospheric pollution on pollen traits In addition, we present the main objectives of this dissertation, as well as its structure;

Chapter 2 - State of the art - this section constitutes a bibliographic review on pollen identification and modifications it suffers after exposure to atmospheric pollutants. It informs about existing works as well as the study methodologies conducted by other authors, both for the pollen identification and for the changes that occur in the various traits of the pollen tested.

Chapter 3 - Article 1 entitled “Testing the Raman parameters of pollen spectra in automatic identification” – in this article we intend to evaluate the ability to use Raman parameters in the identification of airborne pollen. Fifteen pollen species considered to be allergenic were analyzed. After the acquisition, each spectrum was pre-processed and deconvoluted. Only the seven common band intervals for all Raman spectra were chosen to be used in the classification of pollen species using SVM. The revised version of this Article had an outcome of “Minor revisions”.

Chapter 4 – Article 2 entitled “The strong and the stronger: the effects of increasing ozone and nitrogen dioxide atmospheric concentrations in pollen of different forest species” - In this article, we set out to assess the changes that occurred in various pollen traits of four tree species, exposure to polluting gases NO_2 and O_3. Traits such as oxidative stress, viability, soluble protein content, reaction to SOD and changes in the pollen wall evaluated by Raman microscopy. This article will be submitted to the journal Forests.

Chapter 5 - General discussion - in this chapter the general results are explained and correlated. A comparison with the results from other similar studies and implications in the current knowledge on the studied subjects are explored.

Chapter 6 - General conclusions - The main conclusions obtained in this study are presented. This section also includes suggestions for future work.

1. Introduction

1.1. Motivation

Pollen grains are male reproductive structures of seed plants, becoming bioaerosols when released into the atmosphere. Its concentration depends on plant species, geographical distribution, flowering time, pollination type and the environmental conditions affecting its dispersion (Ribeiro and Abreu 2014).

The number of individuals suffering from pollen allergies have grown exponentially in the last decades (Sedghy et al. 2017).This fact is a reason of worry, since the inhabitants affected by this illness have decreased life quality.

Pollen-related allergy reactions are dependent on each individual genetic predisposition, pollen species allergenic potency but also on the expossome. Several studies have been carried out in the last years, in order to fully understand and correlate the mechanisms that take part and may be enhancing pollen allergies.

Airborne pollen calendars are useful to estimate the flowering time of different plants and to indicate the potential allergenic present in the atmosphere at a given time(Ribeiro and Abreu 2014). This type of studies are important, especially as prevention and alert tools for the population suffering of allergies, and automatic real-time airborne pollen monitoring is presently one hot topic in Aerobiology.

Furthermore, pollutant gas emissions, such as NO_2 and O_3, have increased in recent years after the industrial revolution. Several studies correlate allergenic reactions with rural and urban areas and affirm that even with higher quantity of pollen in rural areas, the inhabitants of urban areas have their respiratory allergic symptoms severely enhanced (Schiavoni et al. 2017; Fuertes et al. 2014), .

Air pollutants can have several effects on pollen: alter the physical and chemical characteristics of the pollen surface, it may even cause damage to its wall and release of allergens into the environment; affect biological and reproductive functions by decreasing the pollen fertility or altering the allergenic potential with an increase in health risks (Oduber et al. 2019; Sénéchal et al. 2015).

1.2. Objectives

With this work we intended to grow the knowledge in different areas of expertise and different pollen features. Pollen identifications is one of the first difficulties find to pollen analysis, since until now there's no automatic pollen identification and the traditional method is complex and time consuming. In the first part of this work it's announced a

new approach, using only Raman parameters of few features, to pollen identification. The objective being to have a simpler and faster way to classify pollen species.

In the second part of this work, is presented a very complex and complete analysis of a variety of important pollen features after exposition to environmental pollutants with the objective of understand the changes that happen on pollen grains and the consequences of the changes.

2. State of the art

2.1. Pollen identification

Pollen analysis has been used in many fields of application such as Environmental monitoring (Duque et al. 2015), Agriculture (Fernandez-Gonzalez et al., 2020), Paleobotany (Schopf et al. 2016; Seddon et al. 2019), Forensic Science (Reis et al. 2018) and Medicine (López-vizcaíno et al. 2019; Medek et al. 2019). Traditionally, pollen identification is done using light microscopy, a slow and time-consuming process that requires an experienced observer, since it depends on morphological characteristics of the pollen exine, typically with low taxonomic resolution (Rahl 2008). As such, it is subject to human error.

Samples are usually picked up with some delay. For example, with Hirst, there is a delay of 7 days. This equipment sampling is made through a tape impregnated with silicone, where the pollen adheres, after the air is sucked by the device. The drum rotates slowly allowing for the recording of information per day and per hour. In addition to the natural delay occurring by this form of sample accumulation, there is a need for treatment of the tape and close observation under a microscope for counting and pollen identification. In addition, not being a quick and automatic identification, it is not always possible to identify the species of the pollen grains. For example, the pollen grains belonging to the Poaceae family are practically indistinguishable under the optical microscope.

Several authors are dedicated to the study of pollen identification, since this task would benefit with a faster and more resolved identification of pollen species. Research has been done along the years some with positive and promising results. Techniques such as image-based applications, DNA based techniques, spectrophotometric techniques, etc., are being explored.

Many fields can benefit from a fast and reliable identification. The agriculture field is one of those and may rely on this technique to try to solve many problems. For example, the tomato production is adversely affected by heat stress, and this situation in getting

worse with many countries suffering from heat waves and dry weather. It becomes urgent to have available technologies and advances data science to fast-track the development of heat-tolerant tomato varieties (Ayenan et al. 2020). And, of course, there are applications in health, where identifying allergenic pollen is crucial, for example to give allergic patients the opportunity to manage their own allergies, having local pollen information in real time (Medek et al. 2019). With the climate changes occurring, many more fields will have similar problems, and the need of automatic pollen detection and identifications is now clearer than ever.

2.2. Pollen identification techniques

Image-based applications are one of the more promising techniques being study for pollen identification. The base of this technique is the digital image, being a source of information. in the case of pollen and other biological particles, it is based on microscopic image analysis through image processing detection techniques (France et al. 2000; Ranzato et al. 2007). For automatic image processing, two levels are distinguishable. The first being dedicated to the image acquisition and improvement, and a second one corresponding to an high-level processing dedicated to symbolic image analysis operations, such as description, recognition or interpretation, in order to extract information (Menad et al. 2019). It's possible to obtain some good taxonomic resolution in the identification of some species using image-based techniques, although for other species this technique does not improve the traditional identification technique. With the introduction of texture characterization and/or geometrical features, we may find a better identification, letting an improvement in the classification performance of the distinct pollen types (Manikis et al. 2019; Marcos et al. 2015). More recently, the implementation of real-time automatic pollen recognition systems based on image processing techniques (Oteros et al. 2015) and digital holographic images (Sauvageat et al. 2020), has showed good results in the identification of many pollen species. For this technique, there's still several challenges that need to be addressed, to enhance the present real automatic pollen identification. As an example, grass pollen species, from Poaceae family, are basically indistinguishable in light microscope image. This fact may cause problems to this species identification, due to similarity and that same images are used in image-based techniques.

DNA based techniques are a good alternative, in order to be able to identify grass species, like it has been referred (Kraaijeveld et al. 2015; Brennan et al. 2019). This technique uses the information contained in the DNA to distinguish pollen families, genus and even species. To do this mapping, the first step that involves some complications is the DNA extraction. The air samples analyzed can be collected by standard methods

and DNA extraction occurs afterward following pollen-optimized protocols (Bell et al. 2016; Rojo et al. 2019). Pollen DNA extraction is challenging and is a destructive identification technique, and there are some problems involving pollen abundance quantification (Baksay et al. 2020). Solutions are still needed for these problems. The chloroplastidial and nuclear barcoding markers are commonly used in DNA sequencing according to recent studies.

Other methodologies used in order to achieve automatic pollen identification, are spectrophotometric techniques, such as Fourier transformation infrared spectroscopy (Muthreich et al. 2020; Xu et al. 2018; Zimmermann and Kohler 2014), ultraviolet light induced fluorescence (Forde et al. 2019; Ruske et al. 2018), Raman spectroscopy (Wang et al. 2015) and fluorescent spectroscopy (Mularczyk-Oliwa et al. 2012; Zhang et al. 2019).

The approaches to pollen and other bioaerosols identification have changed and evolved over the time. Initially, the possible objective was to discriminate if the materials present in the atmosphere were biological or non-biological compounds. Presently, the aims became more ambitious by discriminating pollen from other bioaerosols. Fluorescence-based techniques are among the most used since biological/non biological compounds, because of their intrinsic characteristics are easily differentiated, although bioaerosols like pollen and fungal spores are proving more challenging (Forde et al. 2019). This was especially done by UV-LIF, and that idea has evolved to a more elaborate system where the intention has been to distinguish between pollen family and genus. With the objective to determine the best spectroscopic methodology, Ba et al. (2015) compare seven different FTIR and Raman spectroscopy methodologies using the same pollen sample, and conclude that Raman microspectroscopy measurements, that are focused on the corpus region of pollen grains, achieved one of the best taxonomic based differentiation of the pollen.

2.3. Pollen morphology and chemical characteristics of the pollen wall

Inside plants anthers, the reproductive cells suffer many changes and transformations to become pollen grains. Over the development of the different species, pollen grains of angio- and gymnosperm plants differ in number of cells, morphology, cytological and physiological features (Pacini and Franchi 2020). Some of these pollen features such as the size, the stratification and ornamentation of the exine, the intine stratification and composition, color and pollenkitt presence or absence, percentage of water, the type of carbohydrates reserve, and the ability to fertilize (Pacini and Franchi 2020), are used for pollen identification, especially the morphology and composition of the exine.

The pollen wall has elasticity, having perforations and apertures for pollen tube emission. The wall has mainly in its composition a biopolymer named sporopollenin, that is generally waterproof (Blackmore et al. 2007). Exine is subdivided in layers of different chemical composition (Pacini and Franchi 2020), being this fact used by many authors to identify pollen, and it is used to raise the knowledge of pollen chemical composition of the exine or deeper. It is known that pollen can suffer changes due to environmental pollutants. Those changes may occur in various pollen features, the changes can have different intensities to different pollen features and pollen species.

Among the pollutants with the greatest impact on the pollen wall and morphology is particulate matter suspended in the atmosphere. The impacts of this air pollutant on pollen morphology have been studied over the years using mainly optical microscopy or scanning electron microscopy. Some studies report that the particle matter can adhere to the surface of airborne pollen grains, and cause changes in its morphology (Duque et al. 2013; Guedes et al. 2009; Okuyama et al. 2007).

2.4. Raman spectroscopy

Raman spectroscopy has been one of the techniques used by many authors in order to obtain automatic pollen identification (Guedes et al. 2014; Ivleva et al. 2005; Mondol et al. 2019; Schulte et al. 2008; Wang et al. 2015). The recent work of (Mondol et al. 2019), using high-throughput screening is a great development in term of classification algorithms and possible identification of airborne pollen (Doughty and Hill 2020; Guedes et al. 2014) that can allow the increase of single-pollen's spectra resolution and therefore better discrimination of pollen samples.

Raman spectroscopy has some advantage compared to other techniques suggested for pollen identification: it doesn't require sample preparation and it is a nondestructive technique, while giving the possibility to analyze air or aqueous samples with minimal interference (Guedes et al. 2014; Weiss et al. 2019).

The wavenumbers in a Raman spectrum are characteristic and may be attributed to specific chemical compounds which makes it possible to discriminate them. After the deconvolution, with the Raman parameters such as wavenumber and others like the intensity, the integrated intensity and the FWHM (full width at half maximum of the band), remarks to chemical compounds of the pollen wall and can be characteristic for a specific taxon. A diversity of spectra pre-processing techniques can be used in order to reduce noise and enhance the information extracted, as baseline correction, normalization and smoothing (Fukuhara et al. 2019). This bioaerosol has a characteristic of high intensity

spectrum, but with the used of pre-processing techniques it is possible to reduce this effect.

Initially, Raman spectroscopy was used to identify pollen of control samples (known samples) and to separate them from a small number of species and testing different wavelengths to have knowledge of the best one for pollen samples (Ivleva et al. 2005). The attributions of chemical composition to the bands and a family and species correlation that they seem to generate is another important use of Raman spectroscopy for pollen identification and characterization (Schulte et al. 2008). Along the years more pollen species were analyzed, and different algorithms have been tried in order to identify and separate pollen species, by habitat or family/species. Among the techniques/algorithms used, there are supervised and unsupervised machine learning techniques used for classification and precision analysis of the methods (Wang et al. 2015; Zimmermann 2010). Actually, the studies are focused on a faster and automatic pollen identification, where there's still work to be done, but important steps have been made, by the use of high-throughput screening Raman spectroscopy, in order to have automatic data acquisition with reduced human interaction (Mondol et al. 2019).

Raman parameters are obtained after deconvolution of the spectrum. The wavenumber or peak position remarks to chemical structure and identity of the pollen wall, as for the other parameters they define the peak form, them being the intensity, the area under the curve and the full width at half maximum of the peak (FWHM). The Raman parameters are characteristic and may be a simple and reduced information to analyze.

In order to have a fully automatic pollen identification, there is a need of different steps equipment's and functions. Pollen detection system is in first order of need, to a rapid discrimination between pollen grains and all the other particles present in the air flow, biological or nonbiological. However, the detention system and the collection of the data is just a part of an automatic pollen identification protocol, the data analysis/classification is presently one important subject (Okwuashi and Ndehedehe 2020). Many authors have resort to data science/machine learning analysis to improve and automate pollen classification as well as evaluate system detection and analysis capacity.

2.5. Impacts of air pollutants on pollen grains

2.5.1. Air pollutants with greater impact

Several environmental pollutants have been studied in order to understand the effects they can cause, on human, animal, plant and environmental health. In the last decade, the most impacting air pollutants have been tested to understand the changes that are

induced in pollen grains. The pollutants that seem to have more impact are CO_2, NO_X, O_3, SO_2 and particulate matter.

Particulate matter (PM) can be from a natural or anthropogenic source, in the solid or liquid phase (aerosols), of different aerodynamic diameter suspended in the air. Among the natural sources are volcanoes, dust, forest fires, marine aerosol, this last usually find closer to marine environments, burning of fossil fuels in internal combustion engines, thermoelectric and industries among others. Actually, due to their hazard to human health, PMs are monitored with special attention to those with aerodynamic diameters less than 10 µm and 2.5 µm because are inhaled and due to its size can penetrate deeper in the respiratory system (larger particles are filtered in the nose, and progressively smaller particles can go further to the lungs). Human organisms have mechanisms such as mucus, cilia and special cells called macrophages operating to capture particles, however they can cause diseases to the lungs. However, not only human health suffers with this pollutant, the effect for plant species has also been studied (Sgrigna et al. 2020; Šantl-temkiv et al. 2020) and include a reduction of photosynthetic activity.

Carbon dioxide is another gas with great importance in the atmosphere, being essential for life on Earth, being the basic element in the composition of organisms and essential for biomass production. However, with increased concentration over the years as a result of anthropogenic activities, some worries are showed to this gas.

Nitrogen dioxide (NO_2) is one of the seven gases that make up the so-called NO_x, this is a group of highly reactive gases, and the only gas that is regulated by the EPA (Environmental protection agency). This gas has an anthropogenic origin and is very reactive, formed mainly by NO oxidation. The main sources are mainly the burning of fossil fuels, particularly by motor vehicles exhaust and some industrial processes. This gas is not only an important primary air pollutant, but it is also an atmospheric precursor, which contributes to phenomena with a high environmental impact, such as acid rain and eutrophication in the aquatic environment and also the formation of tropospheric ozone. Sulfur dioxide can have a natural source, such as emitted naturally by volcanoes and have an anthropogenic origin in some industrial processes. This gas is harmful to human health and its inhalation can cause severe irritations in the airways as well as enhance previous airways irritations. For the environmental, can be harmful as well this gas is one of the principal components/causes of acid rain along with NO_x, acid rain or acid deposition can be a wet or dry deposition, in terms of rain, fog, snow, hail or even dust that is acidic, prejudicial to the environmental in general. This problem is not restricted to the local, where emissions occur but to everyone, since the wind can make gases travel long distance before the depositions occur, that why these gases are regulated,

their emission control and being diminish along the years in order to reduce acid rains, that are harmful to the environmental, since it causes acidification of water bodies what can cause difficulties to several aquatic species.

Ozone is a pollutant gas with a high oxidative capacity that accelerates the degradation of materials, promotes the loss of plant productivity, increases health problems and the mortality of the exposed population. Tropospheric ozone is generated by the reaction of percussive gases (such as NO_2 or CO) in the presence of solar radiation. Ozone and nitrogen dioxide are among the most mentioned with oxidative capacity and can stimulate, among other reactions in the plant, the generation of reactive oxygen species (ROS) in cells and protein nitrification (Das and Roychoudhury 2014; Shiraiwa et al. 2012).

2.5.2. Pollen features - biological and reproductive functions

The fact that changes occur in the pollen grain after exposure to pollutant gases or particles, is already known, however the extent of the damage and the resulting effects that can result from it are not yet fully known. Thus, several studies have been carried out in recent years, in order to fully understand the mechanisms that change the various characteristics of pollen such as pollen fertility, oxidative stress and protein content. The results found by the various authors are not consensual, in the sense that they do not always report a positive or negative influence of the factors studied, but a variation is mentioned according to the pollen species under analysis and according to the air pollutants to which pollen was exposed and consequently the concentration to which it was exposed directly in the atmosphere or experimentally in vitro.

2.5.2.1. Pollen fertility

The assessment of pollen fertility is important because is related to sexual reproduction and propagation of the species, such as fruit production, in the assessment of intra and interspecific incompatibility or in genetic improvement (Dafni and Firmage 2000).

The pollen fertility is influenced by genetic and environmental factors, including air pollution. The evaluation methodologies usually used to characterize pollen fertility include staining techniques to assess pollen viability and in vitro germination tests or in natural conditions, by the evaluation of the percentage of fertilization and fruit formation (Stanley and Linskens 1974).

Several studies have evaluated pollen fertility after being exposed to air pollutants, both in natural and artificial conditions, and mostly obtained the same conclusion of inducing a decrease in pollen viability or germination (Leghari et al. 2018). Other case

studies report that air pollution can also cause irregularities in the anthers, decreasing their number and end up causing plant infertility (Leghari et al. 2018). This decrease in pollen fertility implies serious losses for future crops, as well as for plant growth, thus affecting production.

As an example, we report the study carried out for *Lepidium virginicus* pollen, after exposure to SO_2 at a concentration of 0.6 ppm during different periods (2, 4 or 8 hours) during flowering season where a 50% decrease in pollen germination was observed in vivo (Du Bay and Murdy 1983). Also, for *Lilium longiflorum* pollen there was an inhibition in the elongation of the pollen tube when the pollen was exposed to a concentration of 0.71 ppm SO_2 or 2 ppm NO_2 (Masaru et al. 1976). Chichiriccò and Picozzi et al. (2007) exposed plants of the *Crocus vernus* species to a concentration of 0.2-11 ppm of NO_2 and verified the marked inhibition of pollen germination while the plant was exposed to the pollutant and a replacement of pollen fertility at the end of the exposure. Pasqualini et al. (2011) reported in pollen from *Ambrosia artemisiifolia* reduction in pollen viability after being exposed to ozone for 5 hours for 7 consecutive days.

Gillespie et al. (2015) described that at relevant ozone levels, negative effects were observed on germination, on the growth of the pollen tube and on the interactions between pollen and stigma, regardless of the time of exposure, for the *Lycopersicon esculentum Mill* species in a combined study in vivo and in vitro.

2.5.2.2. Oxidative stress

Disturbances that occur in the balance between oxidant / antioxidant, in the sense of promoting the oxidant, result in metabolic states that are often called "oxidative stress" (Soares et al. 2018). In biological processes, oxidative stress culminates in the presence of oxidizing agents in large number and they are capable of removing electrons from organic molecules, thus disrupting their functions and damaging essential cellular components such as carbohydrates, lipids, proteins and nucleic acids (Das and Roychoudhury 2014; Soares et al. 2018).

Reactive Oxygen Species (ROS) produced by living beings as a result of normal cellular metabolism, are important in plant signaling, being able to control processes such as growth, development, as well as being essential in the response to abiotic and biotic environmental stimulus of stress, such as air pollution. Subject to a variety of environmental stress from this abiotic, xenobiotic, exposure to heavy metals and herbicide, the generation of ROS includes the formation of two species of free radicals, the superoxide anion ($O_2^{\cdot -}$) and the hydroperoxyl radical ($HO_2^{\cdot}$), and of non-radical

species, the hydrogen peroxide (H_2O_2) and the hydroxyl radical that is highly reactive (OH ·).

In the pollen grain, the regulation of oxidation-reduction reactions (redox potential) are critical for vital factors of cell activation and function, as well as for viability. ROS are synthesized by enzymes such as NADPH oxidase and peroxidases in the cell wall as a result of normal cell metabolism (Speranza et al. 2012).

Ozone is a photo-oxidant gas that is abundant in the atmosphere and is one of the central sources of reactive oxygen species in atmospheric aerosols, and pollen is no exception (Shiraiwa et al. 2012). It was observed by Pasqualini et al. (2011) an increase in NADPH oxidase in *Ambrosia* pollen samples after in vitro exposure to ozone for 7 days. The authors did not find a statistically significant increase in ROS activity in pollen grains, although the increase in NADPH indicates that this enzyme was the main source of the ROS detected.

Despite the differences between the conditions of the studies conducted, of the pollen species, or even the method used to assess the oxidative stress of pollen exposed to pollutants, in general the response to the stress caused is understood as an increase of the presence of ROS and the activity of NADPH oxidase.

2.5.2.3. Protein content

There's a known fact that air pollution, gases or particles, affect pollen grains in various features, being the protein content of pollen one of major importance. In the literature has been frequently reported changes in the content of total soluble proteins, however some disconcordant results pointing out to either an increase, decrease, or even no changes exposure to pollutants (Majd et al. 2004; Pasqualini et al. 2011; Sousa et al. 2012; Rezanejad 2009).

In some studies of Majd et al.(2004), Rezanejad (2009), Rogerieux et al. (2007) it was verified a decrease in the content of soluble proteins in pollen of different species namely *Canna indica*, *Lagerstroemia indica*, *Thuja orientalis* and *Phleum pretense*, respectively, when they compared pollen of polluted areas with unpolluted areas. In this studies there was found significant differences in protein content, being that in the concentrations of soluble proteins per gram of pollen in the polluted areas compared to the control samples, and in the case of *Thuja orientalis* L and *Canna indica*, when control pollen were exposed for 10 and 20 days in the polluted areas, still a decrease in the total protein content was observed.

Some authors have stated an initial increase in pollen protein content in *Argemone mexicana* (Parui et al. 1998) and *Ricinus communis* (Bist et al. 2004) after exposure to

SO_2 when compared to pollen not exposed to this pollutant. These authors also affirmed a decrease in the values of protein content after the first exposure when the pollen was subjected to increasing concentrations of SO_2 and NO_2, as well as for a longer period.

An example of other study, in this case that goes against the previously mentioned, was performed by Ribeiro et al. (2017), where the tendency of increasing the soluble protein content of the pollen exposed to NO_2 was observed, the effects were evaluated for *Platanus x acerifolia* pollen exposed to O_3 and NO_2, and recorded again for the average concentration levels found in the city from Porto. Silva et al. (2015) concluded that exposure to CO_2, a gas whose concentration has been increasing in the atmosphere, negatively affected the total content of soluble proteins as well as the fructose content and pollen fertility.

In contrast, Pasqualini et al. (2011) report no significant changes in protein content of *Ambrosia* pollen exposed to ozone for 5 h per day for 7 consecutive days at a concentration above the limit values established for the protection of human health (0.100 ppm), when compared with unexposed pollen samples.

According to the different results above referend, everything indicates that the effects of air pollutants on the content of soluble proteins in different pollen species, probably depend exactly on that, on the pollen species mentioned, as well as the pollutant gas or particle and the concentration in which it is found. This meaning that there is a possibility that even at a concentration value within legal levels changes may occur, what bring the possibility that for certain plant species the limits may be too high already and actually that may help explain the increase in the number of inhabitants with respiratory allergy as well as the enhance of severe reactions observed in recent decades.

2.6. Allergenic potential

In the last decades, has been verified a grown of the population suffering from pollen-related allergic diseases and respiratory disorders (Pawankar R.et al.2012). This phenomenon has been associated with the interaction between pollen allergen content and air pollution, and there are already some environmental and epidemiological studies carried in this field of research, in order to understand the impact on respiratory allergies on the population (D'Amato 2011).

Some studies done in this field, have proposed that air pollution can modify the pollen's biochemical characteristics among other features, such as fertility and therefore end up increasing the risk of atopic sensitization and exacerbation of allergic symptoms in susceptible individuals (D'Amato 2011; Bosch-Cano et al. 2011).

As it was referred before, the studies carried out by Sousa et al. (2012), Ribeiro and Abreu (2014), report that pollutant gases, such as SO_2, NO_2 and O_3 induce changes in the proteomics of pollen and lead to an exacerbation of respiratory pollen allergies in pollen of the few trees studied even at levels below or approximately equal to the legal limit for the protection of human health according to the European Directive 2008/50 / EC . The work of Sousa et al. (2012), as a concrete example, tested *Acer negundo* pollen, in an environmental in everything similar a real word situation, to different concentrations of NO_2 and SO_2. The results obtained between the samples show different pollen behaviors, however the conclusions are clear, in urban areas, where these gases are present, even in concentrations of SO_2 and NO_2 below the limits established for human protection in Europe, these gases they can indirectly aggravate pollen allergy symptoms and affect plant reproduction.

The study of Reinmuth-Selzle et al. (2017), affirms that an exposure to NO_2 showed only differences that could cause changes in potential allergenic when the contrary was observed in exposure to increasing levels of atmospheric ozone where it was observed changes in allergenic potential and advance, that is the possible cause of the increase in allergies recorded in the last decade. Air pollution is considered by several authors, one of the biggest contributors to the increase in allergies caused by pollen. Numerous studies have already been reported where the protein content has increased and / or report an increase in reactivity to pollen protein extracts exposed to pollutants.

Aiming to evaluate the impacts that can occur to human health such as airway inflammation, pulmonary pathology and imbalance immune, using *Artemisia* pollen exposure of diesel emission, Chen et al. (2020) concluded that the emissions resulting from the exhaust of motor vehicles can increase the allergic response and with consequences on the prevalence of allergies in the inhabitants of the urban environment. Hong et al. (2018), compiles two of the largest atmospheric pollutants, in a study to evaluate the affect in human health, for that it was used specific antibodies, rHum j1 allergen, in order to understand the effects of NO_2 and O_3 on the allergenicity of *Humulus japonicus* pollen, the results showed an increase in the content of this allergen in the pollen what may indicate that the allergenicity has been enhanced. Another examples is the report of Silva et al. (2015) were an increase in the IgE reactivity of sera from patients allergic to the protein extract of *Acer negundo* pollen after exposure of the pollen to increasing levels of CO_2, indicates that the increase in the atmospheric concentration of this gas may negatively influence some characteristics of the pollen, as its fertility and allergic potential.

There are also studies that detect the levels of allergens of some of the main allergenic pollen species present in the atmosphere, for example Bet v 1, Ole e 1, Lol p 1 and Par j 1 and 2, having been observed for example for the atmosphere of Porto high potency allergenic to pollen even though it is at a low level present in the air (Fernandez-Gonzalez et al. 2019).

3. Article 1 - “Testing Raman parameters of pollen spectra in automatic identification.”

Aerobiologia

Testing the Raman parameters of pollen spectra in automatic identification

--Manuscript Draft--

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Abstract:	Pollen identification and quantification is used in many fields of application and research has been conducted to attain accurate automatic pollen recognition aiming reducing the laborious work and subjectivity in human identification. The aim of our study was to evaluate the capacity of the Raman parameters of pollen spectra, calculated for only seven common band intervals in a limited spectral range, to be used as future technique in pollen automatic identification. There were analyzed 15 different pollen species consider to induced allergic reactions. Raman spectra were acquired by an XploRA™ Raman microscope, at an excitation wavelength of 785 nm in a spectral region from 1000 to 1800 cm -1 . After acquisition, each spectrum was pre-processed and deconvoluted using a mixed Gaussian–Lorentzian curve-fitting procedure to determine the Raman parameters: wavenumber, full width at half maximum of the band and integrated intensity. Only seven common band intervals to all Raman spectra, in the fingerprint areas: 1000-1010; 1300-1460 and 1500-1700 cm -1 , were chosen to be used in the classification of the pollen species using SVM (support vector machine). Our results showed that the classification accuracy of all pollen species was 100 % in the training step, while in the testing step 14 out of the 15 pollen species were correctly assigned, including the discrimination between five Poaceae species and between Betula pendula and Corylus avellana . It was also observed that all Raman parameters are important in the classification as well as all wavenumber areas considered. So, our study indicate that the Raman parameters of pollen spectra can be a promising methodology for automatic pollen recognition.
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Testing the Raman parameters of pollen spectra in automatic identification

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Abstract

Pollen identification and quantification is used in many fields of application and research has been conducted to attain accurate automatic pollen recognition aiming reducing the laborious work and subjectivity in human identification.

The aim of our study was to evaluate the capacity of the Raman parameters of pollen spectra, calculated for only seven common band intervals in a limited spectral range, to be used as future technique in pollen automatic identification.

There were analyzed 15 different pollen species consider to induce allergic reactions. Raman spectra were acquired by an XploRA™ Raman microscope, at an excitation wavelength of 785 nm in a spectral region from 1000 to 1800 cm^{-1}. After acquisition, each spectrum was pre-processed and deconvoluted using a mixed Gaussian–Lorentzian curve-fitting procedure to determine the Raman parameters: wavenumber, full width at half maximum of the band and integrated intensity. Only seven common band intervals to all Raman spectra, in the fingerprint areas: 1000-1010; 1300-1460 and 1500-1700 cm^{-1}, were chosen to be used in the classification of the pollen species using SVM (support vector machine).

Our results showed that the classification accuracy of all pollen species was 100 % in the training step, while in the testing step 14 out of the 15 pollen species were correctly assigned, including the discrimination between five Poaceae species and between *Betula pendula* and *Corylus avellana*. It was also observed that all Raman parameters are important in the classification as well as all wavenumber areas considered.

So, our study indicate that the Raman parameters of pollen spectra can be a promising methodology for automatic pollen recognition.

Keywords: Pollen classification; Raman spectra; Spectroscopy; Support Vector Machine

1 Introduction

Pollen analysis has been used in many fields of application such as Environmental monitoring (Ribeiro et al. 2015), Agriculture (Cunha et al. 2016), Paleobotany (Seddon et al. 2019; Schopf et al. 2016), Forensic Science (Orijemie and Israel 2019; Pereira et al. 2020) and Medicine (Lo et al. 2019; Medek et al. 2019). As an example, pollen is one of the most common triggers of season allergic reactions, in some individuals when inhaled causes symptoms due to the proteins that it carries and the numbers of individuals suffering from allergies has grown exponentially in the last years (Sedghy et al. 2017),

Traditionally, pollen identification and quantification is performed manually by light microscope, a process that is time consuming and requires a trained observer to perform it objectively and

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some important pollen types inducing allergies, like *Phleum* or *Dactylis* (García-Mozo 2017) cannot be identify to the genus level. The identification task would benefit with a faster and more resolved identification of pollen species and this is an area where research has been done along the years (Rittenour et al. 2012; Sharma-Ghimire et al. 2019).

Image based applications has been used for pollen identification and other biological particles for a few years (France et al. 2000; Ranzato et al. 2007). It is based on microscopic image analysis through image processing detection techniques and the introduction of texture characterization in the identification has led to improvements in the classification performance of the distinct pollen types (Marcos et al. 2015). More recently, the implementations of real-time automatic pollen recognition systems based on image processing techniques (Oteros et al. 2015) and digital holographic images (Sauvageat et al. 2020), has showed good results in the on-line identification of a number of pollen taxa.

Besides pollen morphological features that provide a good taxonomic distinction at the family, genus and even in some cases at the species level, pollen grains also present several differences concerning molecular features and chemical composition that allow identification (Depciuch et al. 2018; Zimmermann 2018). Recently new methods in pollen identification and quantification have been developed, foreseeing automatic pollen Identification (Šantl-Temkiv et al. 2020).

DNA based techniques have been used for pollen identification and quantification in order to substitute the traditional methodology. The air samples analyze are collected by standard methods and the DNA extraction occurs afterward following optimized methodologies (Rojo et al. 2019; Bell et al. 2016) specially because the pollen DNA extraction is challenging and some problems involving pollen abundance quantification may need other resolution (Baksay et al. 2020). Some studies have showed that this method could provide accurate qualitative identification of grass species (Brennan et al. 2019; Kraaijeveld et al. 2015) that till now are not possible to distinguish with image processing techniques.

Several spectrophotometric techniques have been tested and applied aiming automatic pollen identification. Fourier transformation infrared spectroscopy (FTIR (Muthreich et al. 2020; Xu et al. 2018; Zimmermann and Kohler 2014), ultraviolet light induced fluorescence (UV-LIF)(Ruske et al. 2018; Forde et al. 2019), fluorescent spectroscopy (Mularczyk-Oliwa et al. 2012; Zhang et al. 2019) and RAMAN spectroscopy (Wang et al. 2015). At first, the different approaches were use only to discriminate bioaerosol, and in some cases even pollen, from other materials, biological or not, present in the air, especially the UV-LIF that idea has evolved to a more elaborated system were the intention has been to distinguish between pollen families and genus. Fluorescence-based equipment are being used in the discrimination of materials in the air, non-biological and biological compounds are easily distinguish due to intrinsic characteristics however, bioaerosols like pollen and fungal spores are proving more challenge (Forde et al. 2019). Bağcıoğlu et al. (2015) tested seven different FTIR and Raman spectroscopy methodologies to the same pollen samples and conclude that Raman microspectroscopy measurements, which are focused on the corpus region of pollen grains, achieved one of the best taxonomic based differentiation of pollen.

The detention system and the collection of the data is just a part of an automatic pollen identification protocol, the data analysis/classification (Okwuashi and Ndehedehe 2020) is presently one important subject. Development in data science has given a valuable input into pollen classification based on pollen spectroscopic features. Some studies are using machine learning techniques for classification, as supervised learning were is made a division of the data in training and test, as it happen in SVM (support vector machine), NN (neural networks) or k-nearest neighbors, and others choose unsupervised learning algorithms, here the data is analyzed as one group, as e.g. Hierarchical cluster analysis or k means (Swanson and Huffman 2020).

Independent of the method, the precision of it allow us to conclude about the data in hand, although it is suggested that supervised methods perform better for pollen classification (Seifert et al. 2016; Mondol et al. 2019; Guedes et al. 2014; Schulte et al. 2008).

The use of Raman spectroscopy in the pursuit for automatic pollen identification is not a new research field (Mondol et al. 2019; Wang et al. 2015; Ivleva et al. 2005; Schulte et al. 2008) but recent developments in term of classification algorithms, high-throughput screening (Mondol et al. 2019) and possible identification of airborne pollen (Doughty and Hill 2020; Guedes et al. 2014) can allow the increase of single-pollen's spectra resolution and therefore better discrimination of pollen samples.

Raman spectroscopy is a nondestructive technique, that don't require sample preparation, what comes as an advantage to other techniques suggested for pollen identification and yet is possible to analyze aqueous or air samples with minimal interference (Weiss et al. 2019; Guedes et al. 2014)

Raman spectroscopy evolved along the years, in the beginning was used to identify pollen of known samples (controls) to separate them and in the testing of different wavelengths to ascertain the best suited for pollen (Ivleva et al. 2005). Also, the bands in a Raman spectrum are characteristic and may be assigned to specific chemical compounds which makes it possible to discriminate them. The assignments to the pollen spectrum bands and the correlation to distinct pollen taxa that they seem to generate is another important use of Raman spectroscopy to pollen identification and characterization (Schulte et al. 2008). Pollen grains have a characteristic of high fluorescence spectrum, and that have been a working issue. To enhance the information extracted and reduce noise, a variety of spectrum pre-processing techniques have been used as baseline correction, normalization and smoothing (Fukuhara et al. 2019).

Additionally, Raman parameters obtained after deconvolution of the spectrum such as the wavenumber and other parameters as the intensity, the integrated intensity and the FWHM (full width at half maximum of the band) remarks to chemical compounds of the pollen wall and can be characteristic for a specific taxa.

Therefore, in this work we aim to evaluate the capacity of the Raman parameters of pollen spectra to be used as future technique in pollen automatic identification by simplifying the data acquisition and reducing the volume of information to analyze. Even more because we tested the use of the parameters of only seven common band intervals to all pollen species tested. To pollen classification was used support vector machine with a Data science software.

2 Material and methods

Pollen collection

The pollen samples analyzed by Raman micro-spectroscopy were collected, during the flowering season, in the Porto city, from gardens of the Faculty of Sciences of the University of Porto campus and in public parks. There were analyzed 15 different pollen species from trees, shrubs and weeds (table 1) consider to induced allergic reactions (Galán et al. 2017). Three plants per each species were sampled and flowers/catkins were randomly collected from all quadrants of the plants, in different branches, until a small plastic box was filled. After separation of the anthers from the other plant structures, the anthers were dried at 25◦C during 24 hours, after that time, shivered through different grades of sieves to separate the pollen from the rest of the plant materials and pure pollen were than collect. The samples were stored at−20◦C until analysis (Ribeiro et al. 2017).

Table 1 Pollen analyzed in the study, divided in type of plant and in pollen family.

Family		Pollen species
Trees and shrubs	Aceraceae	*Acer negundo*
	Asteraceae	*Artemisia vulgaris*
	Betulaceae	*Alnus glutinosa*
		Betula pendula
		Corylus avellana
	Cupressaceae	*Cupressus lusitanica*
	Fagaceae	*Quercus robur*
	Oleaceae	*Fraxinus floribunda*
	Platanaceae	*Platanus x acerifolia*
	Salicaceae	*Salix atrocinerea*
Grasses	Poaceae	*Anthoxanthum adoratum*
		Dactylis glomerata
		Holcus lanatus
		Lagurus ovatus
		Lolium perenne

Raman spectra acquisition and processing

Before the analysis, the pollen samples were taken from the storage and left 10 minutes at room temperature.

Raman spectra were acquired by an XploRA™ Raman microscope (Horiba Scientific, France) that combines optical microscopy with a Raman spectroscopy using a laser radiation which allows a "one shot" analysis. A 100x objective lens was used to focus the laser beam on the sample and also to collect the Raman scattered radiation in back-scattering geometry. The Raman signal was detected on a highly sensitive cooled charge-coupled device (CCD) detector to collect the Raman spectra.

Prior to each measurement, the Raman spectrum wavenumber was calibrated using a Si referent standard (520.6±0.1 cm-1). Pollen samples were placed on a glass slide and for each species 3 spectra from three different pollen grains were collected at an excitation wavelength of 785 nm from a Diode laser at a power of 25 mW with a range of diffraction gratings with 1200 lines mm-1 and slit of 300 μm. Extended scans were performed, with five scans of fifty seconds being measured on each pollen grain, in a spectral region from 1000 to 1800 cm^{-1} with approximately 1 cm^{-1} resolution.

Raman spectra were pre-processed involving an automatic polynomial baseline correction to attenuate the fluorescence influence followed by a denoise procedure using the Savitsky-Golay algorithm to increase spectra quality. The spectra were then normalized to a constant area, where the area under the curve is set to 100 (a.u.).

Afterward, each spectrum was deconvoluted using a mixed Gaussian–Lorentzian curve-fitting procedure to determine the precise Raman parameters: wavenumber (W), full width at half maximum of the band (FWHM) and integrated intensity (A). To reduce the influence of the natural variability of the intensity of the spectrum a new parameter was calculated, R_area (pondered area), that being a ratio between the integrated intensity with the total integrated intensity of the deconvolution curve. For the fit of the spectral sets it was used 18 bands, which correspond to the aggregate of principal bands present in the distinct pollen spectra.

The software LabSpec 6 (Horiba Scientific, France) was used for spectra acquisition and deconvolution.

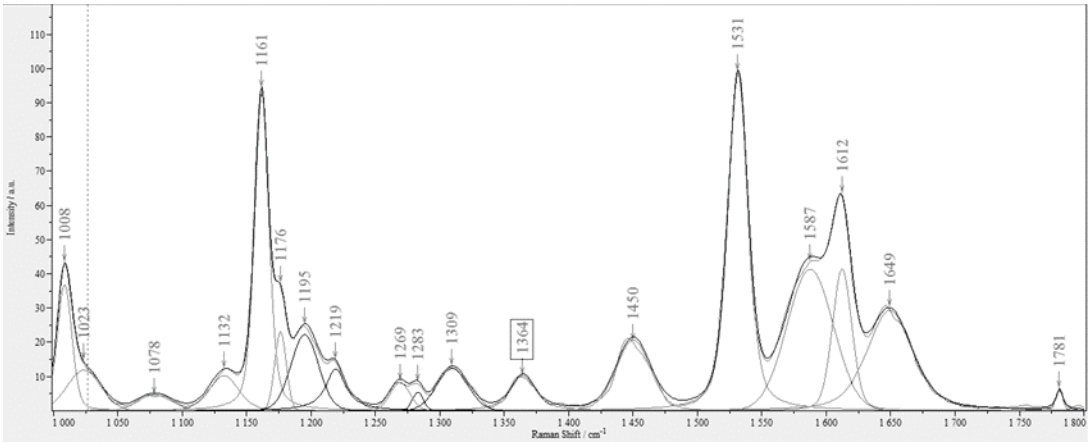

Fig. 1 Example of a spectrum of *Artemisia vulgaris* with a total of 18 bands deconvolution.

Data analysis and pollen classification

A matrix with the preprocessed data was created, where we reunited all the Raman parameters obtained for each of the 18 bands considered in the deconvolution process. Only the seven common band intervals to all Raman spectra were chosen to be used in the classification of the pollen species. The common band intervals were the ones in the fingerprint areas: 1000-1010; 1300-1460 and 1500-1700 cm-1.

For the pollen classification analysis, it was used an open-source software package with tools for data visualization and analysis, data mining and machine learning, Orange 3.24.1. (Demsar et al. 2013).

The potential of Raman parameters to accurately classify the distinct pollen species was evaluated applying a supervised learning algorithm – SVM (support vector machine). SVM is based on the concept of finding a design function that best separates the features in different groups. A hyperplane represents that separation and the best hyperplane is the one that maximizes the distance between features and gives the best classification or regression. This represents a linear classifier, but its usual to find nonlinear separation for the data, so for that, Kernel function are used, the objective remains but the hyperplane adjusts differently to the data. Machine learning algorithms are commonly used for the classification of data, that by training the algorithm with a set of the data and testing the classification with another group of independent data.

To the analysis, the data was divided in two sets, one training group with 66.7% of the spectra (2 per pollen species) and a testing group with 33.3% of the study cases (1 per pollen species).

In Orange, it was selected SVM for classification with a cost value of 1.60 and a regression loss of 0.10, the Kernel function use was RBF (radial basis function) for a nonlinear analysis. That because a pre-analysis of correlation shown that the data were very correlated and a linear classification approach may bias the classification conclusion. The precision of this method for our data was analyzed and a confusion matrix was used to identify the misclassify pollen types.

3 Results and Discussion

Spectra analysis

Raman spectra give information about the pollen chemical characterization, containing specific signals of macronutrients such as lipids, proteins, carbohydrates, water, and even some pigments

(Zimmermann 2010; Schulte et al. 2008; Zimmermann and Kohler 2014; Bağcıoğlu et al. 2015; Pummer et al. 2013; Kendel and Zimmermann 2020; Weglinska et al. 2020). As a result the spectra are quite complex and variable between different genera and even species, which can also be noticed in our results.

The Raman spectra obtained for the 15 different pollen species studied showed distinct 18 bands, that are characteristic of each pollen species, and were selected to be the ones that improve the deconvolution fitting line, in the functionality region between 1000 and 1800 cm^{-1} (Fig.1) but only seven band intervals were common to all studied species, distributed by three fingerprint regions (Fig.2). In fact, the average Raman spectra present some differences between the studied species, being possible to distinguish particularities between the spectra of tree and grass species. The three fingerprint regions are defined by the following seven common band intervals ≈ [1000-1010 cm^{-1}], [1305-1335 cm^{-1}], [1340-1375 cm-1], [1440-1460 cm^{-1}], [1525-1600 cm^{-1}], [1600-1615 cm^{-1}] and [1650-1665 cm^{-1}].

In the 1500-1700 cm^{-1} fingerprint area, a band present in the interval [1525-1600 cm^{-1}] appears in trees mostly at ≈ 1580-1590 cm^{-1}, in the grass species a peak in the same region appears at ≈ 1565 cm^{-1}, and is assigned to nucleic acids (adenine and guanine)(Diehn et al. 2020), the exception is *Anthoxanthum adoratum* with a peak ≈ 1530 cm^{-1}, assigned to carotenoids by Diehn et al. (2020), this is the band with more heterogeneity of peak values.

The tree spectra also present a well-defined strong band in the interval [1600- 1615 cm^{-1}] and one or two medium intensity bands (at 1525-1600 and 1650-1665 cm^{-1}), one before and other after the highest intensity peak, most of times showed as shoulders more or less defined, while in grasses two, three or four medium-low intensity bands are observed in the same region. The band with the higher intensity in all tree spectra, is around ≈ 1608 cm^{-1} and has been assigned to mitochondrial activity but also to the ferulic acid and coumaric acid building blocks in sporopollenin (Diehn et al. 2020). This band is present in all pollen species analyzed, less in *Lolium perenne* were the higher intensity peak appears at ≈ 1600 cm^{-1}, and has been assigned to phenylalanine and tyrosine (Guedes et al. 2014) or to ring stretches of phenyl structures (Ivleva et al. 2005).

A common band observed in the interval [1650- 1665 cm^{-1}] was the one at ≈ 1662 cm^{-1} that has been assigned to vibrations of proteins (Diehn et al. 2020; Schulte et al. 2008) and is present in all pollen species with the exception of *Artemisia vulgaris* pollen where a band is observed in the 1650 cm^{-1} position and may be assigned to Amide I system (C=O) (Guedes et al. 2014; Ivleva et al. 2005).

The fingerprint area between 1300-1460 cm^{-1} can be considered characteristic of grass species where a wide high intensity band is observed in the interval [1340-1375cm^{-1}], with most grass species presenting the peak at ≈ 1370 cm^{-1}, exception is *Dactylis glomerata*. For tree species, this area is quite different, being observed a set of smaller bands around 1360 cm^{-1}, that can be assigned to nucleic acids (adenine and guanine)(Diehn et al. 2020). Also in this fingerprint area (1300-1460 cm^{-1}) two more bands are considered, the peak ≈ 1450 cm^{-1} appears in all species and arises from a deformation made of C-H_2 groups of aliphatic carbon chains (Guedes et al. 2014), the other band is more heterogenous, with a peak at ≈ 1313 cm^{-1} that appears in all trees and in *Dactylis glomerata* pollen and can be assigned to ferulic acid and coumaric acid building blocks in sporopollenin. In the other grass species the peak is shifted to ≈ 1322 cm^{-1}, that is associated to carbohydrates (Diehn et al. 2020).

Finally, in a third fingerprint area (1000-1010 cm^{-1}) a medium to strong band at ≈ 1006 cm^{-1} is characteristic of all pollen and can be assigned to carotenoids (Diehn et al. 2020; Schulte et al. 2008).

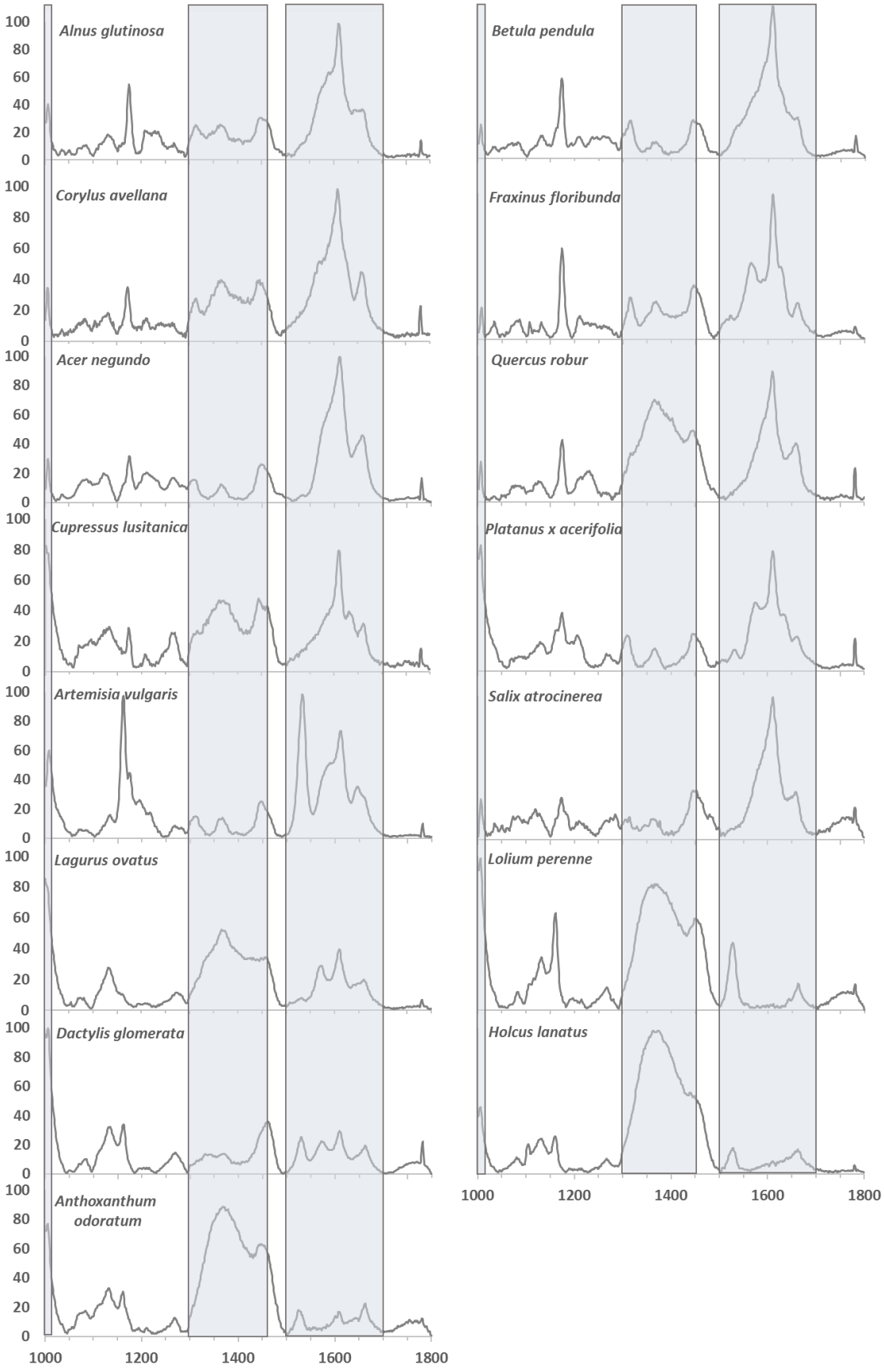

Fig. 2 Average Raman spectra of the 15 pollen species analyzed, and main fingerprint areas marked in the (1000-1010; 1300-1460 and 1500-1700 cm^{-1}).

Classification analysis

The Raman spectra complexity and variability between distinct pollen types also enables its identification and classification, by applying data analysis, to taxonomic levels that are many times impossible by humans under light microscopy (Kraaijeveld et al. 2015; Mondol et al. 2019). In our study it was assessed the possibility of the Raman parameters of the seven common bands to all pollen species to be sufficient for the classification process, a different approach to what has been usually done in other studies that use information of the full or reduced spectral range.

It was evaluated the classification potential in three combinations, all 15 tested species datasets, of only tree species and finally of only grass species. The best classification possible of these data sets is achieved when it was used the Raman parameters of the wavenumber (W), full width at half maximum of the band (FWHM) and integrated intensity (A) (table 2). In our study, the R_area (pondered area) parameter did not improved the classification.

The classification performance using all pollen species was very high, being perfect in the training step with a classification accuracy (CA) of 100%, a precision and recall of 100%, while in the testing step 14 out of the 15 pollen species were correctly assigned (precision of 90%; and CA and recall of 93.3%). The exception was *Salix atrocinerea* pollen, which was misclassified as *Acer negundo*. It was possible to perform the distinction between pollen from *Betula pendula* and *Corylus avellana*, two taxa belonging to the same family presenting very similar morphologies, which can pose some challenges for the image processing-based methods minimized by their desynchronized presence in the atmosphere (Sauvageat et al. 2020).

Table 2 – Confusion matrix resulted from the SVM analysis on test step of the Raman parameters (wavenumber, full width at half maximum of the band and integrated intensity) of the seven common wavenumbers from the Raman spectra of the pollen from 15 plant species.

Classification %	*Acer negundo*	*Alnus glutinosa*	*Betula pendula*	*Corylus avellana*	*Cupressus lusitanica*	*Fraxinus floribunda*	*Platanus x acerifolia*	*Quercus robur*	*Salix atrocinerea*	*Artemisia vulgaris*	*Anthoxanthum a.*	*Dactylis glomerata*	*Holcus lanatus*	*Lagurus ovatus*	*Lolium perenne*
Acer negundo	**100**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alnus glutinosa	0	**100**	0	0	0	0	0	0	0	0	0	0	0	0	0
Betula pendula	0	0	**100**	0	0	0	0	0	0	0	0	0	0	0	0
Corylus avellana	0	0	0	**100**	0	0	0	0	0	0	0	0	0	0	0
Cupressus lusitanica	0	0	0	0	**100**	0	0	0	0	0	0	0	0	0	0
Fraxinus floribunda	0	0	0	0	0	**100**	0	0	0	0	0	0	0	0	0
Platanus x acerifolia	0	0	0	0	0	0	**100**	0	0	0	0	0	0	0	0
Quercus robur	0	0	0	0	0	0	0	**100**	0	0	0	0	0	0	0
Salix atrocinerea	**100**	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0
Artemisia vulgaris	0	0	0	0	0	0	0	0	0	**100**	0	0	0	0	0
Anthoxanthum a.	0	0	0	0	0	0	0	0	0	0	**100**	0	0	0	0
Dactylis glomerata	0	0	0	0	0	0	0	0	0	0	0	**100**	0	0	0
Holcus lanatus	0	0	0	0	0	0	0	0	0	0	0	0	**100**	0	0
Lagurus ovatus	0	0	0	0	0	0	0	0	0	0	0	0	0	**100**	0
Lolium perenne	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**100**

Comparing our results with those obtained by other authors, where the full or reduced spectral range was used in the pollen discrimination (Diehn et al. 2020; Ivleva et al. 2005; Boris Zimmermann and Kohler 2014), it is possible to see that the Raman parameters allowed attaining as good classification. As observed by Schulte et al. (2008), even though pollen taxa related to

the genus and family level present chemical similarities, which are indicative of both phylogenetic relationship and mating behavior, in our study it was possible to discriminate between the distinct pollen species.

Using the information of only seven common band intervals, we are able to reduce the volume of data necessary to classify the pollen species as well as the time of analysis and spectra acquisition due to the small spectral region studied.

Table 3 shows the confusion matrix for the tree pollen species classification corresponding to the test step.

Table 3 Confusion matrix resulted from the SVM analysis on test step of the Raman parameters (wavenumber, full width at half maximum of the band and integrated intensity) of the seven common wavenumbers from the Raman spectra of the pollen from tree plant species.

Classification %	*Acer negundo*	*Alnus glutinosa*	*Betula pendula*	*Corylus avellana*	*Cupressus lusitanica*	*Fraxinus floribunda*	*Platanus x acerifolia*	*Quercus robur*	*Salix atrocinerea*
Acer negundo	**100**	0	0	0	0	0	0	0	0
Alnus glutinosa	0	**0**	0	0	0	0	0	**100**	0
Betula pendula	0	0	**100**	0	0	0	0	0	0
Corylus avellana	0	0	0	**100**	0	0	0	0	0
Cupressus lusitanica	0	0	0	0	**100**	0	0	0	0
Fraxinus floribunda	0	0	0	0	0	**100**	0	0	0
Platanus x acerifolia	0	0	0	0	0	0	**100**	0	0
Quercus robur	0	0	0	0	0	0	0	**100**	0
Salix atrocinerea	**100**	0	0	0	0	0	0	0	**0**

The trees CA declined a little compared when all species were used but the majority were accurately classified. The exceptions were *Salix atrocinerea* that remains misclassified as *Acer negundo* and now *Alnus glutinosa* is classified as *Quercus robur*. For this analysis we obtained a CA and a recall of 77.8% and a precision value of 66.7%. As it happens for the total species SVM analysis, in the train step the CA, recall and precision value were 100%.

It is interesting to observe that *Alnus* pollen was correctly discriminated when all studied species were considered. When we use only the Raman parameters of common band in tree pollen, we are distinguishing among more similar spectra. The Raman spectrum of *Quercus robur* pollen in the 1300-1460 cm^{-1} fingerprint areas has much more similarities to the grass spectra and therefore when grasses are included in the training step the *Q. robur* would be set further from the tree species.

When only grass species are tested, the classification renders the best performance with all species being correctly classified in both the training and testing steps (table 4), with a CA, recall and precision value of 100%.

Through high-throughput screening Raman spectroscopy (HTS-RS), Mondol et al. (2019) used the Raman spectra fingerprint region (758–1800 cm^{-1}) from pollen of 15 genera belonging to the Poaceae family and applied PCA-SVM for their classification. The predictions among Poaceae genera was high (around 79% accuracy, and sensitivity of 80%), but the number of pollen grains/species analyzed was much higher when compared with our study.

Table 4 Confusion matrix resulted from the SVM analysis of the Raman parameters (wavenumber, full width at half maximum of the band and integrated intensity) of the seven common band intervals from the Raman spectra of the pollen from 5 grass species.

Classification %	*Anthoxanthum a.*	*Dactylis glomerata*	*Holcus lanatus*	*Lagurus ovatus*	*Lolium perenne*
Anthoxanthum adoratum	**100**	0	0	0	0
Dactylis glomerata	0	**100**	0	0	0
Holcus lanatus	0	0	**100**	0	0
Lagurus ovatus	0	0	0	**100**	0
Lolium perenne	0	0	0	0	**100**

We tested also if the Poaceae pollen Raman peaks observed in the fingerprint area 1300-1460 cm^1, with distinct spectral features among the tested species, could be enough for a correct classification among them. It was observed that it was not sufficient for a good classification, in the train step the value of accuracy and recall was 80% and the precision of 83.3% where *Anthoxanthum a*. was misclassify as *Holcus l.*, and *Holcus l*. as *Lagurus o.*.

Distinction among airborne Poaceae genera, or even species if possible, is important in terms of pollen-related allergy issues. Grass species are one of the most common and higher allergenic species and their wide distribution around the globe as well as number of species, causing several allergic reactions in susceptible individuals (García-Mozo 2017). However, not all grass species induce allergies, but a few genera like *Lolium* spp., *Dactylis* spp., *Anthoxanthum* spp., *Phleum* spp., among others, are the most allergic ones (Brennan et al. 2019; García-Mozo 2017). With an extensive flowering season, that lasts around 4 to 5 months between March-July and September, and with several annual peaks in airborne pollen concentration it makes months of suffering for grass pollen allergen suffers (Ribeiro and Abreu 2014). Presently, the grass airborne pollen season is not discriminated by the different genera or species, and the pollen season of the most allergenic ones may be common to other type of pollen season what may enhance the allergic individual reaction (García-Mozo 2017). So, it becomes clear the importance to identify the different airborne pollen contributors, and among the Poaceae it is a real challenge to exactly defined the traits of the flowering seasons, beginning and ending, for the different genera (Brennan et al. 2019). In fact, the morphological similarities among the airborne pollen from the different Poaceae genera makes almost impossible their distinction. Features such as number of apertures, shape and texture are quite similar, posing great analytical challenges to image-processing algorithms (Ronneberger et al. 2002), although Poaceae pollen morphology is so typical that are easily distinguished among other airborne non-Poaceae pollen. With our study it was possible to distinguish between 5 different species of Poaceae by using the Raman parameters of only seven common band intervals from the full pollen Raman spectrum.

In our study we also tested the contribution of each Raman parameter or their different combinations in pollen classification (Fig.3a).

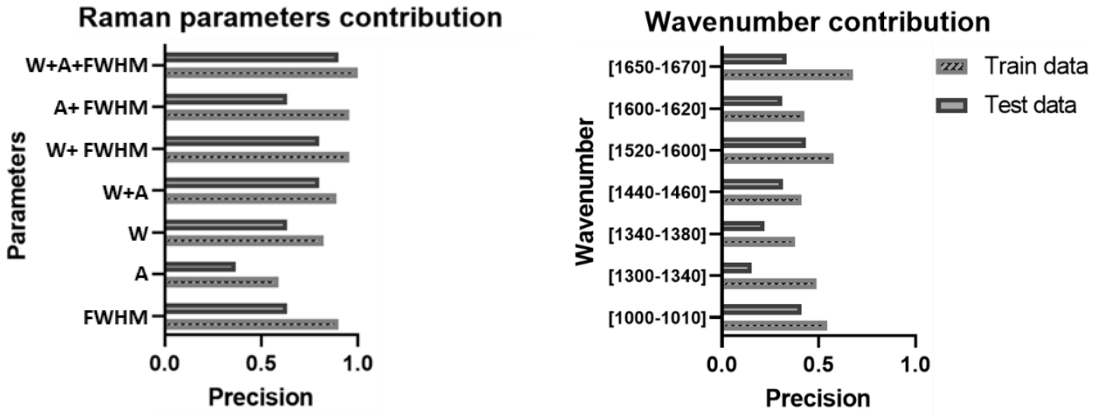

Fig. 3 Contribution of each Raman parameter (Wavenumber (W), full width at half maximum of the band (FWHM) and integrated intensity (A)) or their combination (graphic on the right) and each of the 7 common band intervals (graphic on the left) in the pollen classification performance. Values of the precision obtained using SVM analysis in orange software with the same configuration used in the classification of the species. Both train and test sets of data were analyzed.

It was observed that the combination of two or more parameters gave better results than using only a single parameter. In the table below Fig. 3 we can see the precision values obtained in the training and testing steps.

Considering each Raman parameter alone, the integrated intensity (A) is the one that less contributes to the classification (in training: 37% and in testing: 59%). The wavenumber (W) and full width at half maximum of the band (FWHM) showed equal precision in the testing step (63%) but the W achieved best classification in the train step (90%).

When the parameters are combined in groups of two, the W + FWHM gave better performance than the other combinations. However, only the combination of all parameters allows the best classification with 100% CA in the training step and 90% in the test one.

Finally, with the question in mind if all the 7 common wavenumbers are important to the discrimination of the pollen from the 15 studied species and therefore avoid overfitting of the classification algorithm, we tested removing the parameter's data of each wavenumber considered at a time without any other change in the remaining data. It was observed that all wavenumbers are important for the correct classification of the pollen species. CA in the training step and even less in the test one was very low when any wavenumber is removed (Fig. 3).

One interesting observation was when we test the removal of only the last band interval [1650-1670 cm^{-1}], the discrimination in the train step is negatively affected although in a smaller percentage (precision value of 94%) when compared with the removal of the other intervals.

The results shift from no misclassification, to 50% wrong classification for a few species but all trees (*Alnus glutinosa* pollen misclassify as *Betula pendula*; *Salix atrocinerea* as *Acer negundo*; *Corylus avellana* as *Fraxinus angustifolia*) being still possible to make distinction between the tree and the grass species. This behavior was not observed when all the other intervals were removed at a time and grasses were misclassified as trees and vice-versa. So, the band interval [1650-1670 cm^{-1}], seems to contribute to the classification between trees and grasses as well as among the different tree species.

All parameters are important in the classification, the wavenumber values are one of the most important though this parameter alone can be tricky. The calibration made in the equipment it is basically a calibration of the wavelength, and that if not taken as a routine can induce differences in this parameter. Zimmerman et al. (2014) described small shifts in the wavenumber position,

even in pollen spectra of the same species in different geographical regions. In fact, in our study, in the test group the performance of this parameter alone is not good.

The proposed methodology in our study could be a promising approach for Raman-based automatic pollen classification, however one drawback in the small dataset used in the training and testing of the classification algorithm. It will be interesting to test in the future the efficiency of the seven common band intervals in discriminating between the studied pollen types by the use of a high-throughput analysis methodology.

4 Conclusion

Our study focused on testing the possibility of using the band Raman parameters: wavenumber (W), full width at half maximum of the band (FWHM) and integrated intensity (A) instead of the all spectrum into pollen classification. All parameters are important in the classification, with the wavenumber alone contributing the most to the classification.

The results obtained proved to be possible using the Raman parameters of seven band intervals, common to all pollen types, to achieve a successful classification of different pollen species. Fourteen out of 15 pollen species were discriminated including some that are morphologically very difficult or even impossible to identified by the human eye e.g between five Poaceae species and between two species of Betulaceae, as *Betula pendula* and *Corylus avellana*.

It would be interesting to further test the proposed methodology using a larger number of species, including fresh pollen and more Poaceae species, as well as the minimal acquisition time to still achieve a precise classification.

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References

Bağcıoğlu, M., Zimmermann, B., & Kohler, A. (2015). A Multiscale Vibrational Spectroscopic Approach for Identification and Biochemical Characterization of Pollen. *PLOS ONE, 10*(9), e0137899, doi:10.1371/journal.pone.0137899.

Baksay, S., Pornon, A., Burrus, M., Mariette, J., Andalo, C., & Escaravage, N. (2020). Experimental quantification of pollen with DNA metabarcoding using ITS1 and trnL. *Scientific Reports, 10*(1), 4202, doi:10.1038/s41598-020-61198-6.

Bell, K. L., de Vere, N., Keller, A., Richardson, R. T., Gous, A., Burgess, K. S., et al. (2016). Pollen DNA barcoding: current applications and future prospects. *Genome, 59*(9), 629-640, doi:10.1139/gen-2015-0200.

Brennan, G. L., Potter, C., de Vere, N., Griffith, G. W., Skjoth, C. A., Osborne, N. J., et al. (2019). Temperate airborne grass pollen defined by spatio-temporal shifts in community composition. *Nature Ecology & Evolution, 3*(5), 750-754, doi:10.1038/s41559-019-0849-7.

Cunha, M., Ribeiro, H., & Abreu, I. (2016). Pollen-based predictive modelling of wine production: application to an arid region. *European Journal of Agronomy, 73*, 42-54, doi:https://doi.org/10.1016/j.eja.2015.10.008.

Demsar, J., Curk, T., Erjavec, A., Gorup, C., Hocevar, T., Milutinovic, M., et al. (2013). Orange: Data Mining Toolbox in Python. *Journal of Machine Learning Research, 14*(35), 2349−2353.

Depciuch, J., Kasprzyk, I., Drzymała, E., & Parlinska-Wojtan, M. (2018). Identification of birch pollen species using FTIR spectroscopy. *Aerobiologia, 34*(4), 525-538, doi:10.1007/s10453-018-9528-4.

Diehn, S., Zimmermann, B., Tafintseva, V., Seifert, S., Bağcıoğlu, M., Ohlson, M., et al. (2020). Combining chemical information from grass pollen in multimodal characterization. *Fronteirs in Plant Science, 10*(1788), doi:10.3389/fpls.2019.01788.

Doughty, D. C., & Hill, S. C. (2020). Raman spectra of atmospheric particles measured in Maryland, USA over 22.5 h using an automated aerosol Raman spectrometer. *Journal of Quantitative Spectroscopy and Radiative Transfer, 244*, 106839, doi:https://doi.org/10.1016/j.jqsrt.2020.106839.

Forde, E., Gallagher, M., Walker, M., Foot, V., Attwood, A., Granger, G., et al. (2019). Intercomparison of multiple UV-LIF spectrometers using the aerosol challenge simulator. *Atmosphere, 10*(12), 797.

France, I., Duller, A. W. G., Duller, G. A. T., & Lamb, H. F. (2000). A new approach to automated pollen analysis. *Quaternary Science Reviews, 19*(6), 537-546, doi:https://doi.org/10.1016/S0277-3791(99)00021-9.

Fukuhara, M., Fujiwara, K., Maruyama, Y., & Itoh, H. (2019). Feature visualization of Raman spectrum analysis with deep convolutional neural network. *Analytica Chimica Acta, 1087*, 11-19, doi:https://doi.org/10.1016/j.aca.2019.08.064.

Galán, C., Dahl, A., Frenguelli, G., & Gehrig, R. (2017). Airborne pollen in Europe. In *Allergy and Allergen Immunotherapy: New Mechanisms and Strategies* (pp. 127-162).

García-Mozo, H. (2017). Poaceae pollen as the leading aeroallergen worldwide: A review. *Allergy, 72*(12), 1849-1858, doi:10.1111/all.13210.

Guedes, A., Ribeiro, H., Fernández-González, M., Aira, M. J., & Abreu, I. (2014). Pollen Raman spectra database: Application to the identification of airborne pollen. *Talanta, 119*, 473-478, doi:https://doi.org/10.1016/j.talanta.2013.11.046.

Ivleva, N. P., Niessner, R., & Panne, U. (2005). Characterization and discrimination of pollen by Raman microscopy. *Analytical and Bioanalytical Chemistry, 381*(1), 261-267, doi:10.1007/s00216-004-2942-1.

Kenđel, A., & Zimmermann, B. (2020). Chemical analysis of pollen by FT-Raman and FTIR spectroscopies. *Fronteirs in Plant Science, 11*(352), doi:10.3389/fpls.2020.00352.

Kraaijeveld, K., de Weger, L. A., Ventayol García, M., Buermans, H., Frank, J., Hiemstra, P. S., et al. (2015). Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. *Molecular Ecology Resourses, 15*(1), 8-16, doi:10.1111/1755-0998.12288.

Lo, F., Bitz, C. M., Battisti, D. S., & Hess, J. J. (2019). Pollen calendars and maps of allergenic pollen in North America. *Aerobiologia, 35*(4), 613-633, doi:10.1007/s10453-019-09601-2.

Marcos, J. V., Nava, R., Cristóbal, G., Redondo, R., Escalante-Ramírez, B., Bueno, G., et al. (2015). Automated pollen identification using microscopic imaging and texture analysis. *Micron, 68*, 36-46, doi:https://doi.org/10.1016/j.micron.2014.09.002.

Medek, D. E., Simunovic, M., Erbas, B., Katelaris, C. H., Lampugnani, E. R., Huete, A., et al. (2019). Enabling self-management of pollen allergies: a pre-season questionnaire evaluating the perceived benefit of providing local pollen information. *Aerobiologia, 35*(4), 777-782, doi:10.1007/s10453-019-09602-1.

Mondol, A. S., Patel, M. D., Rüger, J., Stiebing, C., Kleiber, A., Henkel, T., et al. (2019). Application of high-throughput screening Raman spectroscopy (HTS-RS) for label-free

identification and molecular characterization of pollen. *Sensors, 19*(20), 428, doi:10.3390/s19204428.

Mularczyk-Oliwa, M., Bombalska, A., Kaliszewski, M., Włodarski, M., Kopczyński, K., Kwaśny, M., et al. (2012). Comparison of fluorescence spectroscopy and FTIR in differentiation of plant pollens. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 97*, 246-254, doi:https://doi.org/10.1016/j.saa.2012.05.063.

Muthreich, F., Zimmermann, B., Birks, H. J. B., Vila-Viçosa, C. M., & Seddon, A. W. R. (2020). Chemical variations in Quercus pollen as a tool for taxonomic identification: Implications for long-term ecological and biogeographical research. *Journal of Biogeography, 47*(6), 1298-1309, doi:10.1111/jbi.13817.

Okwuashi, O., & Ndehedehe, C. E. (2020). Deep support vector machine for hyperspectral image classification. *Pattern Recognition, 103*, 107298, doi:https://doi.org/10.1016/j.patcog.2020.107298.

Orijemie, E. A., & Israel, I. (2019). Palynomorphs and travel history of vehicles in Nigeria. *Aerobiologia, 35*(3), 497-510, doi:10.1007/s10453-019-09577-z.

Oteros, J., Pusch, G., Weichenmeier, I., Heimann, U., Möller, R., Röseler, S., et al. (2015). Automatic and online pollen monitoring. *International Archives of Allergy and Immunology, 167*(3), 158-166, doi:10.1159/000436968.

Pereira, J. S. R., Ribeiro, H., & Abreu, I. (2020). Spatial and temporal environmental pollen analysis of footwear worn in the area of Barcelos, North-West Portugal, in a forensic context. *Aerobiologia, 36*(1), 89-94, doi:10.1007/s10453-019-09598-8.

Pummer, B. G., Bauer, H., Bernardi, J., Chazallon, B., Facq, S., Lendl, B., et al. (2013). Chemistry and morphology of dried-up pollen suspension residues. *Journal of Raman Spectroscopy, 44*(12), 1654-1658, doi:10.1002/jrs.4395.

Ranzato, M., Taylor, P. E., House, J. M., Flagan, R. C., LeCun, Y., & Perona, P. (2007). Automatic recognition of biological particles in microscopic images. *Pattern Recognition Letters, 28*(1), 31-39, doi:https://doi.org/10.1016/j.patrec.2006.06.010.

Ribeiro, H., & Abreu, I. (2014). A 10-year survey of allergenic airborne pollen in the city of Porto (Portugal). *Aerobiologia, 30*(3), 333-344, doi:10.1007/s10453-014-9331-9.

Ribeiro, H., Costa, C., Abreu, I., & Esteves da Silva, J. C. G. (2017). Effect of O3 and NO2 atmospheric pollutants on Platanus x acerifolia pollen: Immunochemical and spectroscopic analysis. *Science of The Total Environment, 599-600*, 291-297, doi:https://doi.org/10.1016/j.scitotenv.2017.04.206.

Ribeiro, H., Guimaraes, F., Duque, L., Noronha, F., & Abreu, I. (2015). Characterisation of particulate matter on airborne pollen grains. *Environmental Pollution, 206*, 7-16, doi:10.1016/j.envpol.2015.06.015.

Rittenour, W. R., Hamilton, R. G., Beezhold, D. H., & Green, B. J. (2012). Immunologic, spectrophotometric and nucleic acid based methods for the detection and quantification of airborne pollen. *Journal of Immunological Methods, 383*(1-2), 47-53, doi:10.1016/j.jim.2012.01.012.

Rojo, J., Núñez, A., Lara, B., Sánchez-Parra, B., Moreno, D. A., & Pérez-Badia, R. (2019). Comprehensive analysis of different adhesives in aerobiological sampling using optical microscopy and high-throughput DNA sequencing. *Journal of Environmental Management, 240*, 441-450, doi:10.1016/j.jenvman.2019.03.116.

Ronneberger, O., Schultz, E., & Burkhardt, H. (2002). Automated pollen recognition using 3D volume images from fluorescence microscopy. *Aerobiologia, 18*(2), 107-115, doi:10.1023/A:1020623724584.

Ruske, S., Topping, D. O., Foot, V. E., Morse, A. P., & Gallagher, M. W. (2018). Machine learning for improved data analysis of biological aerosol using the WIBS. *Atmospheric Measurements Techniques, 11*(11), 6203-6230, doi:10.5194/amt-11-6203-2018.

Šantl-Temkiv, T., Sikoparija, B., Maki, T., Carotenuto, F., Amato, P., Yao, M., et al. (2020). Bioaerosol field measurements: Challenges and perspectives in outdoor studies. *Aerosol Science and Technology, 54*(5), 520-546, doi:10.1080/02786826.2019.1676395.

Sauvageat, E., Zeder, Y., Auderset, K., Calpini, B., Clot, B., Crouzy, B., et al. (2020). Real-time pollen monitoring using digital holography. *Atmospheric Measurements Techniques, 13*(3), 1539-1550, doi:10.5194/amt-13-1539-2020.

Schopf, J. W., Calça, C. P., Garcia, A. K., Kudryavtsev, A. B., Souza, P. A., Félix, C. M., et al. (2016). In situ confocal laser scanning microscopy and Raman spectroscopy of bisaccate pollen from the Irati Subgroup (Permian, Paraná Basin, Brazil): Comparison with acid-macerated specimens. *Review of Palaeobotany and Palynology, 233*, 169-175, doi:https://doi.org/10.1016/j.revpalbo.2016.03.004.

Schulte, F., Lingott, J., Panne, U., & Kneipp, J. (2008). Chemical Characterization and Classification of Pollen. *Analytical Chemistry, 80*(24), 9551-9556, doi:10.1021/ac801791a.

Seddon, A. W. R., Festi, D., Robson, T. M., & Zimmermann, B. (2019). Fossil pollen and spores as a tool for reconstructing ancient solar-ultraviolet irradiance received by plants: an assessment of prospects and challenges using proxy-system modelling. *Photochemical and Photobiological Sciences, 18*(2), 275-294, doi:10.1039/c8pp00490k.

Sedghy, F., Sankian, M., Moghadam, M., Ghasemi, Z., Mahmoudi, M., & Varasteh, A. R. (2017). Impact of traffic-related air pollution on the expression of Platanus orientalis pollen allergens. *International Journal of Biometeorology, 61*(1), 1-9, doi:10.1007/s00484-016-1186-z.

Seifert, S., Merk, V., & Kneipp, J. (2016). Identification of aqueous pollen extracts using surface enhanced Raman scattering (SERS) and pattern recognition methods. *Journal of Biophotonics, 9*(1-2), 181-189, doi:10.1002/jbio.201500176.

Sharma Ghimire, P., Tripathee, L., Chen, P., & Kang, S. (2019). Linking the conventional and emerging detection techniques for ambient bioaerosols: a review. *Reviews in Environmental Science and Bio/Technology, 18*(3), 495-523, doi:10.1007/s11157-019-09506-z.

Swanson, B. E., & Huffman, J. A. (2020). Pollen clustering strategies using a newly developed single-particle fluorescence spectrometer. *Aerosol Science and Technology, 54*(4), 426-445, doi:10.1080/02786826.2019.1711357.

Wang, C., Pan, Y.-L., Hill, S. C., & Redding, B. (2015). Photophoretic trapping-Raman spectroscopy for single pollens and fungal spores trapped in air. *Journal of Quantitative Spectroscopy and Radiative Transfer, 153*, 4-12, doi:https://doi.org/10.1016/j.jqsrt.2014.11.004.

Weglinska, M., Szostak, R., Kita, A., Nems, A., & Mazurek, S. (2020). Determination of nutritional parameters of bee pollen by Raman and infrared spectroscopy. *Talanta, 212*, 120790, doi:10.1016/j.talanta.2020.120790.

Weiss, R., Palatinszky, M., Wagner, M., Niessner, R., Elsner, M., Seidel, M., et al. (2019). Surface-enhanced Raman spectroscopy of microorganisms: limitations and applicability on the single-cell level. *Analyst, 144*(3), 943-953, doi:10.1039/C8AN02177E.

Xu, X.-l., Zheng, Y.-z., Chen, X.-c., Zhu, F.-l., & Miao, X.-q. (2018). Identification of cattail pollen, pine pollen and bee pollen by Fourier transform infrared spectroscopy and two-

dimensional correlation infrared spectroscopy. *Journal of Molecular Structure, 1167*, 78-81, doi:https://doi.org/10.1016/j.molstruc.2018.04.076.

Zhang, M., Klimach, T., Ma, N., Könemann, T., Pöhlker, C., Wang, Z., et al. (2019). Size-resolved single-particle fluorescence spectrometer for real-time analysis of bioaerosols: Laboratory evaluation and atmospheric measurements. *Environmental Science & Technology, 53*(22), 13257-13264, doi:10.1021/acs.est.9b01862.

Zimmermann, B. (2010). Characterization of pollen by vibrational spectroscopy. *Applied Spectroscopy, 64*(12), 1364-1373, doi:10.1366/000370210793561664.

Zimmermann, B. (2018). Chemical characterization and identification of Pinaceae pollen by infrared microspectroscopy. *Planta, 247*(1), 171-180, doi:10.1007/s00425-017-2774-9.

Zimmermann, B., & Kohler, A. (2014). Infrared spectroscopy of pollen identifies plant species and genus as well as environmental conditions. *PLOS ONE, 9*(4), e95417, doi:10.1371/journal.pone.0095417.

4.Article 2 – “The strong and the stronger: the effects of increasing ozone and nitrogen dioxide atmospheric concentrations in pollen of different forest species.

forests

MDPI

Article

The strong and the stronger: the effects of increasing ozone and nitrogen dioxide atmospheric concentrations in pollen of different forest species

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Abstract: This is the abstract section, about **300 words maximum**. For research articles, abstracts should give a pertinent overview of the work. We strongly encourage authors to use the subheadings given. *Research Highlights:* Place the novelty of the content and highlight the significance of the study. *Background and Objectives:* Place the question addressed in a broad context and highlight the purpose of the study. *Materials and Methods:* Describe briefly the main methods or treatments applied, including the study population description. *Results:* Summarize the article's main findings. *Conclusions:* Indicate the main conclusions or interpretations.

Keywords: pollen; forest; oxidative stress; proteins; cultures; viability

1. Introduction

Forest ecosystem is one of the most diverse in nature providing food, resources, energy and services that benefits humans, animals, as well as the economy and the environment [1], [2]. Urban forests and peri-urban forests are not new concepts although their importance to the urban society is now clearer than ever, with climate change as well with the increasing pollution problems, solutions or mitigations are in order to problems, such as heat waves and extreme weather events. Nature based solutions can present a better technology and should be primary choice to reduce and prepare cities to the impacts of climate change. Urban and peri-urban forests are suggest to improve life quality providing physical and physiological well-being [3], [4], [5], also providing water and soil purification and management of waterflow in storms and extreme climate events [6], improving air quality [7], [8],for carbon storage and sequestration [9] and as climate regulator and that as energy saver.

Forest ecosystems are dynamic systems, not static, when exposed to stress conditions they can suffer disturbances in their balance. The impact on the ecosystem depends on several factors, but it can include reduction of production and biomass, alteration in the composition of species or community structure, increase in plagues and diseases and increase in morbidity [10], and also a

change in the natural activities of the forests that are useful, such as climate and hydrological control, as well as prevention of soil erosion.

Air pollution has reached worrying levels, especially in certain urban areas of the planet, and forests play a decisive role in reducing air pollution [11]. In the past decades globally, tropospheric ozone concentrations have increased [12], even with a slightly decrease being observed in NOx concentration assign to environmental regulations.

Pollutants are emitted from all sources in a region, with time they mix and diffuse from the emission spot, and undergo physical, chemical and photochemical reactions, the regional air pollutants have been documented for the potential impact in forests, especially oxidant pollutants, like ozone and NOx as well as acid depositions, mostly from nitric and sulfuric acids [10]. Ozone is a secondary air pollutant, being synthetized in the atmosphere in the presence of chemical precursors, in this case hydrocarbon and nitrogen oxides, and because of this it may be detected anywhere. NOx is an anthropogenic pollutant mostly from burning of fossil fuels, especially in motorized vehicles and some industrial processes [13], even being mostly released in urban areas, this pollutant can travel long distances and react to form different species of pollutants [14].

Some studies report that significant plant functions can be compromised in the face of high concentrations of air pollutants. A disturbance in reactive oxygen species (ROS) leads to a physiological situation of the plant called oxidative stress, which can affect its growth and development and even cause the cells death [15], [16]. The photosynthetic efficiency of the plants may be decreased in polluted zones [15], also high degree of air pollution can cause irregularities in the anthers, decrease their number and end up causing male sterility [17]. There is a wide variety of studies carried out on the impacts of pollution in forest plant traits [18], as leaf [19], fruit [20], photosynthetic activity in this field [21], and the vast majority suggest the occurrence of significant effects after exposure to environmental pollutants such as O_3 and NO_2.

Among plant features one important for successful reproduction are pollen grains. During flowering season pollen is released in high quantity from the plants and carried by air, animals or water to perform the pollination, that way allowing fruit formation and future seeds. Therefore pollen quality is important in ensuring the forest survival, growth and production, as well as for animal feeding, such as bees and other insects, that have in pollen an important source of protein and nutrients.

When pollen is released by the plants, it can become a biological aerosol [22], which can undergo changes in its biological traits [23]; [24]. Pollutants have been reported to affect pollen at morphologic and physiological levels, the size of the pollen grain, the quantity of pollen produced has been reported to suffer changes as well as decrease in pollen viability after exposure to exhaust pollution [17]. Air pollutants can have several effects on pollen: (a) changes in the physical and chemical characteristics of the surface pollen (b) affect biological and reproductive functions: pollen viability and germination, (c) changes in allergenic potential with consequences of increased health risks, d) changes in oxidative stress and d) alter nutritional value ([25], [26] [27], [28], [29], [30], [31], [32]). However, among the studies performed to ascertain the effects of air pollution on pollen traits results sometimes are contradictory and there is the suggestion that different species have different reactions to the pollutant but as well to distinct exposure concentrations. But to our knowledge there is no study comparing several forest species at the same experimental conditions to prove this point. In this study we focus on four tree species: *Betula pendula, Corylus avellana, Acer negundo* and *Quercus robur* that are common forest trees, but also present in urban sites as well. We aimed to investigate the effect that may occur in pollen fertility, oxidative stress, protein content and wall composition after exposure to ozone and NO_2 at limit concentration levels for vegetation protection in Europe

(2008/50/EC). The knowledge of pollen sensitivity and tolerance to stress factors such air pollution is of key importance for forest sustainability ensuring most efficient production with highest benefits and lowest resource losses.

2. Materials and Methods

2.1. Pollen sampling

The pollen samples analyzed in this study were collected directly from the different trees during their flowering season. Three plants per each species were sampled and flowers/catkins were randomly collected from all quadrants of the plants, in different branches. After separation from the other plant structures, the anthers were dried at 25◦C during 24 hours, after that time, shivered through different grades of sieves to separate the pollen from the rest of the plant materials and pure pollen was collected and stored at−20◦C until analysis [28].

2.2. Exposure experiments od pollen to pollutant gasses

Individual samples of 50 mg of pollen, were exposed in Falcon tubes open in both sides and covered with a fine mesh with a pore opening size of 23 μm (SEFAR PET 1000), which were placed inside a fumigation chamber, under measured temperature and humidity conditions, for 6 hours. In the chamber, in order to imitate what happens in the atmosphere during a day, a Solar Simulator (Newport Oriel 96,000 150W), a fans (SUNON SF23080AF) to homogenize the air, temperature and relative humidity sensors (EBI20 sensor) and gas sensors (AEROQUAL Series 500 sensors; O_3 sensor range: 0–0.5 ppm, NO_2 sensor range: 0–0.2 ppm) were set inside the chamber as describe in [30].

The samples were submitted to both pollutant gas (Table 1) having as reference the legal limit value for the protection of vegetation, as given in the EU Ambient Air Quality Directive 2008/50/EC, corresponding to approximately half of the legal limit, the legal limit value and the double legal limit value.

Table 1. Average and standard deviation values of temperature, relative humidity and gas concentrations obtained inside the fumigation chamber for each assay.

	Blank	**½ L. O_3**	**Lim O_3**	**2xL. O_3**	**½ L. NO_2**	**Lim NO_2**	**2xL. NO_2**
Acer negundo							
Δ T (ºC)	24.5 ±0.391	25.4 ±1.554	25.2 ±0.236	23.7 ±0.905	25.3 ±0.424	24.6 ±0.645	24.2 ±0.259
Δ RH (%)	59.1 ±0.654	61.2 ±1.641	61.9 ±0.968	61.4 ±1.274	62.3 ±0.241	61.2 ±1.238	60.2 ±0.427
[Gas] (ppm)	---	0.031 ±	0.064 ±0.014	0.125 ±0.016	0.051 ±0.010	0.113 ±0.037	0.197 ±
Betula pendula							
Δ T (ºC)	25.6 ±0.880	25.6 ±0.975	21.5 ±1.182	24.6 ±1.080	28.4 ±0.706	25.6 ±0.680	25.4 ± 0.866
Δ RH (%)	59.6 ±0.991	60.7 ±0.932	59.1 ±1.398	60.9 ±0.944	58.2 ±1.098	60.3 ±1.310	59.8 ±1.285
[Gas] (ppm)	---	0.030	0.061	0.012	0.055	0.104	0.195

		±0.001	±0.015	±0.030	±0.021	±0.017	±0.036
Corylus avellana							
Δ T (ºC)	24.4 ±0.643	25.3 ±0.899	24.8 ±0.714	24.6 ±0.628	25.0 ±0.868	25.3 ±0.645	24.0 ±0.882
Δ RH (%)	59.9 ±0.925	56.2 ±1.802	60.9 ±2.010	59.9 ±1.000	57.4 ±0.377	57.4 ±1.527	63.2 ±0.575
[Gas] (ppm)	---	0.030 ±0.005	0.059 ±0.013	0.119 ±0.090	0.057 ±0.022	0.105 ±0.044	0.203 ±0.060
Quercus robur							
Δ T (ºC)	24.0 ±0.979	24.9 ±1.529	25.5 ±1.103	25.0 ±1.577	24.6 ±0.923	24.6 ±0.903	24.9 ±0.557
Δ RH (%)	60.7 ±2.218	61.4 ±1.407	58.5 ±1.568	62.1 ±1.670	59.0 ±1.189	61.2 ±1.572	61.0 ±1.157
[Gas] (ppm)	---	0.029 ±0.010	0.060 ±0.017	0.120 ±0.015	0.059 ±0.018	0.112 ±0.027	0.220 ±0.046

Δ T: average temperature registered along the experiment; Δ RH: : average relative humidity registered along the experiment; [Gas]:average gas concentration

To obtain these average concentration values, control injections of NO_2 and O_3 were during the exposure time, both environmental condition and gas values were register during the time of exposure. An A2ZS-1GLAB ozone system was used to generate ozone, connected to a timer (OMRON H3DK-S1) to control the gas injection. The NO_2 was obtained by chemical reaction of concentrated nitric acid and solid copper mixed in a sealed bottle, at stoichiometric amounts [33]. For the blank sample, pollen was subjected to the same conditions as the remaining samples, but without exposure to any gas.

2.3. Pollen viability

Pollen viability was assayed using a fluorochromatic reaction with fluorescein diacetate (FDA) method as described by Heslop-Harrison (1984) [34]. If the pollen vegetative cell membrane is intact, the fluorescein diacetate enters in the cell and associates with enzymes (esterases) inducing fluorescence temporarily [34]. Viable pollen grains appear with strong fluorescence while non-fluorescence pollen grains are non-viable.

The pollen grains were suspended in a FDA solution (2 mg/ml in PBS) during 15 min in obscurity and were then centrifuged for 3 minutes at 4 000 rpm. The supernatant was discarded, deionized water was added and placed on ice in order to stop the reaction.

To calculate the pollen viability, the counting of viable grains was carried out using a fluorescence microscope (DMLB; Leica Microsystems, Wetzlar, Germany) equipped with a mercury lamp of 50 W and a filter for blue light. Three random fields per sample (each one containing 100 pollen grains) were considered and the viability percentage expressed as average percentage rate of the 3 counts.

2.4. Pollen protein extraction and quantification

Pollen samples (10 mg) were suspended in Eppendorf tubes with 12 mg of zirconia beads (0.5 nm) and 200 µL of phosphate buffered saline (1:20 w/v) at pH 7.4 and 10 µl of protease inhibitor cocktail (Sigma).Then, each tube was stirred using a Mini-BeadbeaterTM at 4ºC (3 cycles each with

the duration of 30 sec. with pause intervals of 15 sec. at 5000 rpm). Subsequently, the samples were kept at 4ºC under constant (orbital) stirring for 2 hours, centrifuged twice (16,100 g for 20 minutes at 4ºC) and the supernatant was filtered (0.45 μm Millipore filter) and centrifuged again in the conditions previously described.

The soluble protein content of all pollen extracts were quantified by means of a colorimetrical reaction with the Coomassie Protein Assay Reagent (sigma-Aldrich) in microtiter plates (300μL/well at 530nm) according to the Bradford method [35]. Different BSA concentrations (0-2000 μg/ml) were used to estimate a standard curve for protein calibration.

2.5. Pollen oxidative stress analysis

2.5.1. Detection of Reactive Oxygen Species (ROS)

The detection of ROS was determined using the fluorescent ROS indicator dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH2-DA). To each pollen sample (2 mg) it was added 100 μL of 2.5 mM DCFH2-DA in PBS, kept in the obscurity for 15 minutes at 25ºC and then centrifuged for 3 minutes at 4 000 rpm, the supernatant was discarded, and PBS was added.

This step was repeated, fresh PBS medium was added, and the content of ROS was determined using a fluorescence microscope (DMLB; Leica Microsystems, Wetzlar, Germany). A 450-490 mm excitation filter and a 515 emission filter were used. The percentages of fluorescent pollen grains relative to the total pollen grains was determined per each sample counting three random fields, containing 100 pollen grains each.

2.5.2. NADPH oxidase enzymatic activity

NADPH oxidase activity was quantified by spectrophotometry, based on the nitro blue tetrazolium assay (NBT). The pollen samples (1mg/assay) were hydrated in PBS solution (1mL), agitated gently for 30 min at 4ºC and centrifuged at 7300g for 3 min. After that the supernatant was discarded and was added 2mM nitroblue tetrazolium (NBT) with and without nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) (1mMol/L). The samples were then incubated at 37 °C for 30 min, washed with PBS and centrifugated (7300g for 3 min). To dissolve the formazan precipitate, it was added pure methanol and the samples were agitated at room temperature for 20 min followed by centrifugation (7300 g for 3 min). Then a PBS centrifugation (7300 g for 3 minutes) was made, so the final samples was clear. Finally, the absorbance of the supernatants was measured, in triplicate, in a microtiter plate at 530nm (200μL/well).

2.5.3. Western blotting analysis of Superoxide dismutase (SOD)

Proteins were separated under reducing conditions in 12.5% (w/v) polyacrylamide gels (10 μg of pollen protein extracts per lane) and were electroblotted (TE22 Mighty small transpher unit - GE Healthcare) onto nitrocellulose membranes (Protran, Whatman® Schleicher & Schuell, Germany) using transfer buffer (192 mM glycine, 25 mM Tris and 20% methanol) during 2 h at 200 mA. The blots were blocked in TBS-T (20 mM Tris, 150 mM NaCl with 0.1% Tween-20) with 5%defatted milk for 1 h. Afterwards were incubated overnight at 4 °C with a customized pollen anti-Cu/Zn SOD Ab (sequence accession nº EU250769.1) diluted 1:2500 in TBS-T solution [36].

After incubation overnight, the membranes were washed with TBS-T and immunodetection was performed using goat anti-rabbit IgG Ab HRP conjugated (Thermo Fisher Scientific, USA) (1:2000) for one hour. Protein bands were detected using an ECL solution (Luminata™ Crescendo,

Western HRP substrate), visualized in a Chemidoc™ XRS + system (Bio-rad laboratories) and quantified using the ImageLab™ v5.2 software. The changes in the reactivity to the different pollen extracts were evaluated through the optic density values of the reactive bands.

2.6. Raman microspectroscopy

For spectra acquisition it was used XploRA™ Raman microscope (Horiba Scientific, France) that combines optical microscopy with a Raman spectroscopy using a laser radiation which allows a "one shot" analysis. A 100x objective lens was used to focus the laser beam on the sample and also to collect the Raman scattered radiation in back-scattering geometry. The Raman signal was detected on a highly sensitive cooled charge-coupled device (CCD) detector to collect the Raman spectra.

The wavenumber of the Raman spectrum, before each use, was calibrated with Silicon referent standard (520.6±0.1 cm-1). For the acquisition, it was used an excitation wavelength of 785 nm from a Diode laser at a power of 25 mW with a range of diffraction gratings with 1200 lines mm-1 and slit of 300 μm. The pollen samples, stored at -20 ºC, were kept 10 minutes at room temperature before being distributed on a glass slides and from each sample 4 spectra from four different pollen grains were collected. Extended scans were performed, with five scans of fifty seconds being measured on each pollen grain, in a spectral region of 1000 to 1800 cm-1 with approximately 1 cm-1 resolution.

For spectral acquisition and pre-processing, the software Labspec 5 was used. All spectra were processed with an automatic polynomial baseline correction to attenuate the fluorescence influence followed by a noise reduction using the Savitsky-Golay algorithm in order to increase spectra quality. The spectra were then normalized to a constant area, where the area under the curve is set to 100 (a.u.). To obtain a lower signal-to-noise ratio, average spectra for each sample were calculated and compared using the KnowItAll ® Software.

2.7. Statistical analysis

The results obtained in the different experiments are presented as means± standard deviation and the Shapiro-Wilk test was used to test the normality of the data. To evaluate the quantitative effect of the O_3 and NO_2 pollutant gases an one-way ANOVA was applied followed by the Turkey post hoc test when significant differences were detected to perform comparisons. Statistical analysis was conducted using a Microsoft Office Excel 2013 spreadsheet and IBM SPSS statistics version 24 (IBM, USA).

3. Results

3.1. Pollen viability

In the figure 1 are shown the results of viability rates of *A. negundo, B. pendula, C. avellana and Q. robur* pollen before and after exposure to pollutants. Pollen viability of non-exposed pollen ranged between 76.3±2.1% for *C. avellana*, 72.0±2.0% for *Q. robur*, 69.3±0.6% for *B. pendula* and 68.7±1.2% for *A. negundo*.

The overall trend pointed to a significant statistical reduction ($p<0.05$) in pollen viability induced by O_3 and NO_2 but the percentage loss varied depending on pollen species, gas tested and their concentrations, that loss can be evaluated in table 2, for all the samples that presented a significative loss.

Table 2. Average percentage of viability lost with signaling to represent that, for *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe.

	1/2 O_3 Lim	O_3 Lim	O_3 2xLim	1/2 NO_2 Lim	NO_2 Lim	NO_2 2xLim
Acer negundo	≈	≈	≈	≈	7% ↘	9% ↘
Betula pendula	≈	9% ↘	8% ↘	≈	8% ↘	17% ↘
Corylus avellana	12% ↘	21% ↘	13% ↘	5% ↘	13% ↘	16% ↘
Quercus robur	4% ↘	5% ↘	7% ↘	3% ↘	7% ↘	12% ↘

The highest decrease in pollen viability was registered for *C. avellana* (average of 16% when exposed to O_3 and 11% to NO_2) followed by *B. pendula* (average of 7% when exposed to O_3 and 8% to NO_2), *Q. robur* (average of 5% when exposed to O_3 and 7% to NO_2) and finally *A. negundo* (average of 5% to NO_2).

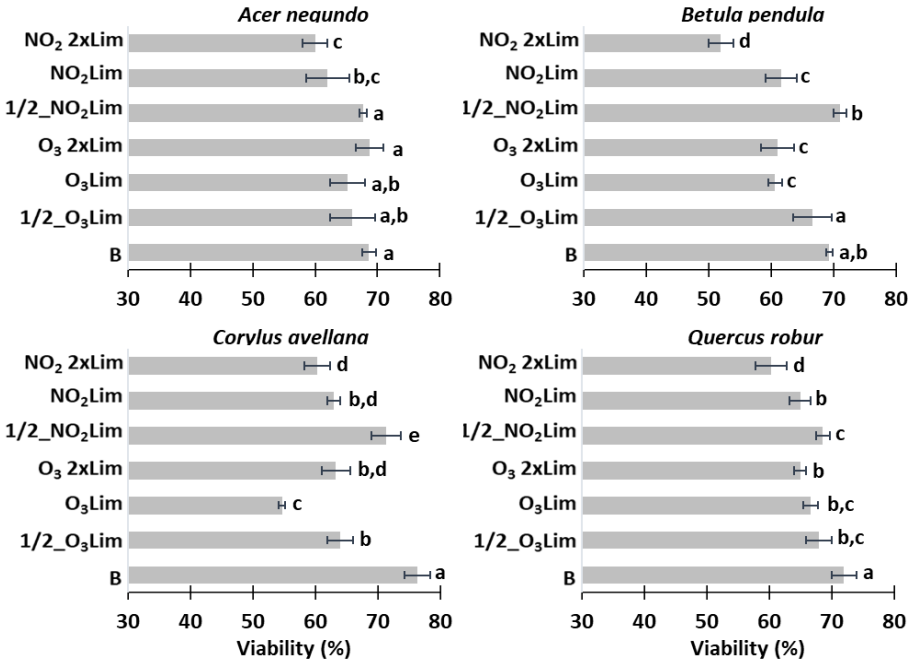

Figure 1. Average and standard deviation in pollen viability of *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe. Different letters indicate statistically significant differences given by the ANOVA test followed by the Tukey's pos-hoc test ($p<0.05$).

For *C. avellana and Q. robur* pollen, regardless of the NO_2 concentration a significant decrease in pollen viability was observed while for *A. negundo* and *B. pendula*, only the limit and the double legal limit value for the protection of vegetation induced a significant reduction.

Regarding the O_3 influence in pollen viability, no significant changes was observed for *A. negundo*, for *B. pendula* only the limit and the double legal limit concentrations induced a significant

238 decrease while for *C. avellana and Q. robur* O_3 had always a significant negative effect on pollen
239 viability.
240

241 *3.2. Pollen total soluble protein content*

242 The content of total soluble protein (TSP) in the different pollen species was quite distinct and
243 ranged between 8536.5±3.15 µml for *A. negundo*, 7241.9±173.9 µml for *Q. robur*, 3256.2±64.1 µml for *C.*
244 *avellana* and 1410.9±64.6 µml for *B. pendula* (Fig. 2). In table 3, it's represented the overall tendency of
245 all pollen species studied.
246
247 **Table 3.** Average percentage of TSP content with signaling to represent increase and decrease, for *A.*
248 *negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at
249 concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim)
250 values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air
251 quality and cleaner air for Europe.

	1/2 O_3 Lim	O_3 Lim	O_3 2xLim	1/2 NO_2 Lim	NO_2 Lim	NO_2 2xLim
Acer negundo	13% ↘	18% ↘	22% ↘	19% ↘	8% ↘	19% ↘
Betula pendula	≈	22% ↗	≈	≈	≈	≈
Corylus avellana	20% ↘	10% ↗	≈	≈	16% ↗	28% ↗
Quercus robur	≈	19% ↘	13% ↘	29% ↘	≈	53% ↘

252

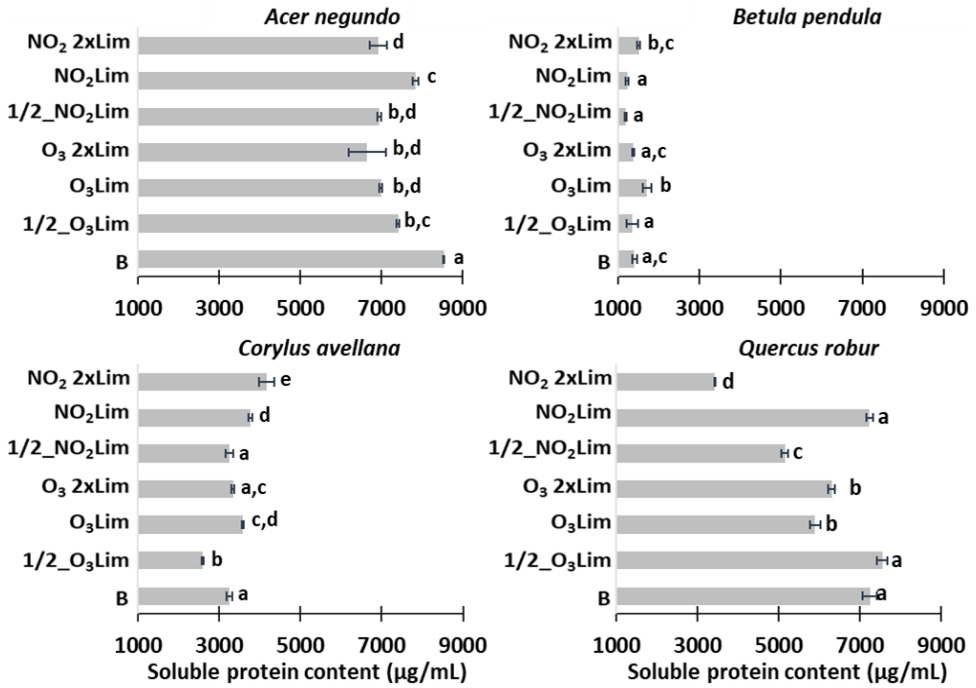

Figure 2. Average and standard deviation in pollen soluble protein content of *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe. Different letters indicate statistically significant differences given by the ANOVA test followed by the Tukey's pos-hoc test ($p<0.05$)

253 *A. negundo* pollen registered a significant decrease in TSP after pollutant exposure ($p<0.05$)
254 compared with the blank sample, but the influence observed amongst the distinct treatments (O_3 vs

NO_2) was not consistently statistically significant. A significant decrease in TSP was also observed for *Q. robur* pollen but only in the pollen samples exposed to O_3 at half the limit value and NO_2 at twice the limit values for the protection of vegetation.

For *B. pendula*, there were observed either an increase or decrease in TSP content compared with the blank sample however these differences were not consistently statistically significant. For *C. avellana*, either no change or a significant increase in TSP after exposed to NO_2 at the limit and twice the limit values and at O_3 limit value concentration. Exception was the O_3 concentration corresponding to half the limit value that induced a decrease in TSP. If we do not consider the results of the statistical analysis, these two pollen species show a similar pattern in the TSP increase and decrease towards the distinct gas concentrations tested.

3.3. Pollen oxidative stress

3.3.1. Detection of reactive oxygen species (ROS)

The ROS baseline rate detected of the four pollen species analyzed was very distinct, being the lowest observed for *A .negundo* (14.3±2.1%), followed by *B. pendula* and *Q. robur* with similar percentages (25.7±1.5 and 25.0±2.7) and the highest was registered for *C. avellana* (45.7±1.5%).

Table 4. Average percentage of ROS with signaling meaning significative increase for *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe.

	1/2 O_3 Lim	O_3 Lim	O_3 2xLim	1/2 NO_2 Lim	NO_2 Lim	NO_2 2xLim
Acer negundo	12% ↗	13% ↗	12% ↗	12% ↗	12% ↗	23% ↗
Betula pendula	≈	6% ↗	3% ↗	≈	2% ↗	6% ↗
Corylus avellana	≈	12% ↗	≈	≈	≈	7% ↗
Quercus robur	8% ↗	12% ↗	11% ↗	≈	12% ↗	10% ↗

The overall trend pointed to an increase in ROS percentage after pollen exposure to the pollutants (table 4) although not always statistically significant for *B. pendula* and *C. avellana*. The highest average increase was observed for *A. negundo* (14%) and the lowest for *B. pendula* (3%).

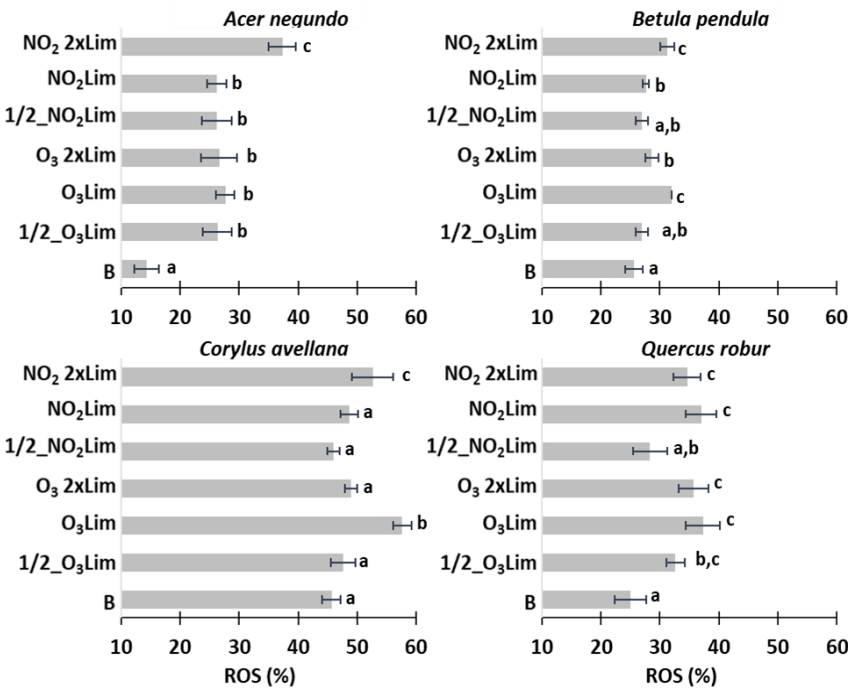

Figure 3. Average and standard deviation in pollen reactive oxygen species (ROS) content of *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe. Different letters indicate statistically significant differences given by the ANOVA test followed by the Tukey's pos-hoc test ($p<0.05$).

Observing the results (figure 3) of each pollen species individually and considering the differences among pollutant's treatments, for *A. negundo* pollen no significant differences in ROS percentage were observed between the distinct concentrations of O_3 and NO_2, exception was pollen exposed to NO_2 at twice the limit values for the protection of vegetation that induced a significant increase in ROS. Similar behavior was observed for *Q. robur* pollen, although in this case, the exceptions were for pollen exposed to concentrations of O_3 and NO_2 corresponding to half the limit value that induced a significantly lower increase in ROS when compared with the other pollutant's treatments. For *B. pendula*, pollen exposure to O_3 and NO_2 at half the limit value did not induce a significant increase in ROS percentage, all other concentrations did. Contrariwise, for *C. avellana* only O_3 levels at the limit values and NO_2 at twice the limit value for vegetation protection induced a significant increase in ROS percentage.

3.3.2. NADPH oxidase enzymatic activity

Opposite to the trends observed for the other pollen traits tested, overall NADPH oxidase enzymatic activity (hereafter referred to as NADPH) was the parameter with the lowest change induced by the pollutant gases, being towards the increase in the enzymatic activity (table 5).

Table 5. Average percentage of NADPH oxidase enzymatic activity with signaling meaning significative increase for *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe.

	1/2 O_3 Lim	O_3 Lim	O_3 2xLim	1/2 NO_2 Lim	NO_2 Lim	NO_2 2xLim
Acer negundo	≈	≈	≈	≈	≈	28% ↗
Betula pendula	15% ↗	≈	≈	≈	15% ↗	14% ↗
Corylus avellana	≈	≈	≈	≈	≈	≈
Quercus robur	21% ↗	≈	≈	≈	≈	20% ↗

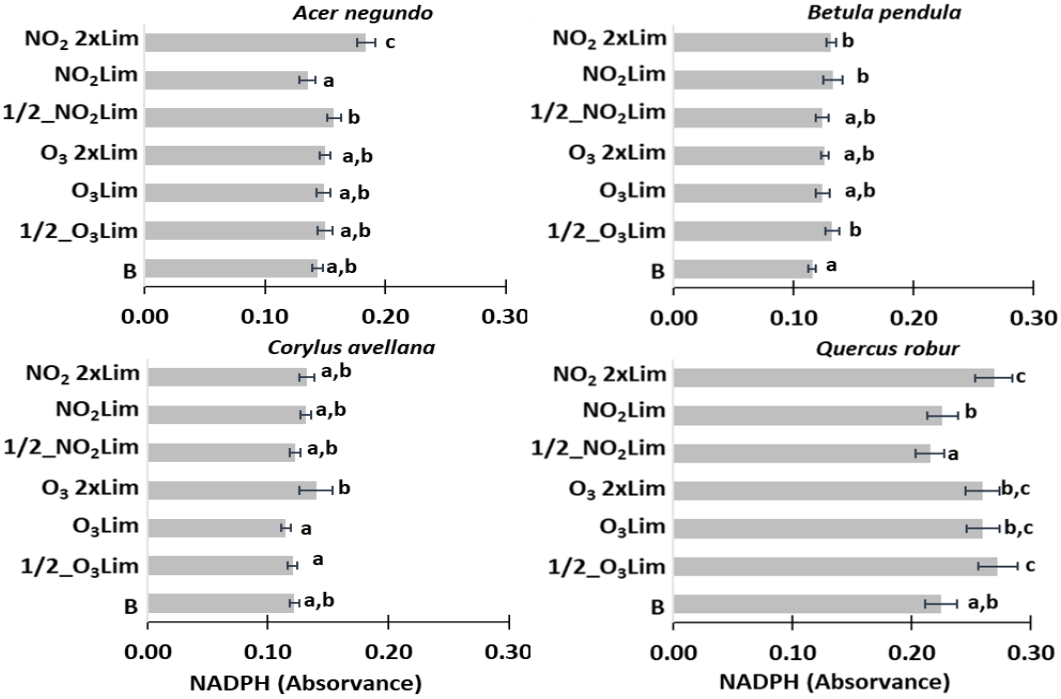

Figure 4. Average and standard deviation in pollen NADPH oxidase enzymatic activity in *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe. Different letters indicate statistically significant differences given by the ANOVA test followed by the Tukey's pos-hoc test ($p<0.05$).

As showed in figure 4, *C. avellana* pollen NADPH activity was not significantly influenced by none of the gasses tested while for *A. negundo* and *B. pendula* only the pollen exposed to the two highest NO_2 concentration levels presented a significant statistical change ($p<0.05$) in NADPH activity. Ozone only appears to influence NADPH activity in *Q. robur* pollen.

3.3.3. Superoxide dismutase (SOD) expression

SOD (superoxide dismutase) isoforms expressed in each pollen species trough immunoblotting analysis using specific antibody can be observed in figure 5. Differences in expression induced by O_3 and NO_2 were observed and varied depending not only on the isoform nature, but also among the same isoform varied between pollen species and gas tested.

Not all pollen species presented the same SOD isoforms. On the basis of their theoretical molecular weights the bands around 15 KDa (peroxisomal CuZn-SOD), 35-36 KDa (chloroplastidic Fe-SOD) were common to all species and the one around 25 KDa (mitochondrial Mn-SOD) was only absent in A. negundo. One band around 45-46 KDa, absent only in B. pendula, and 60 KDa, present only in A. negundo, can be assigned to multimeric forms of the CuZn-SOD.

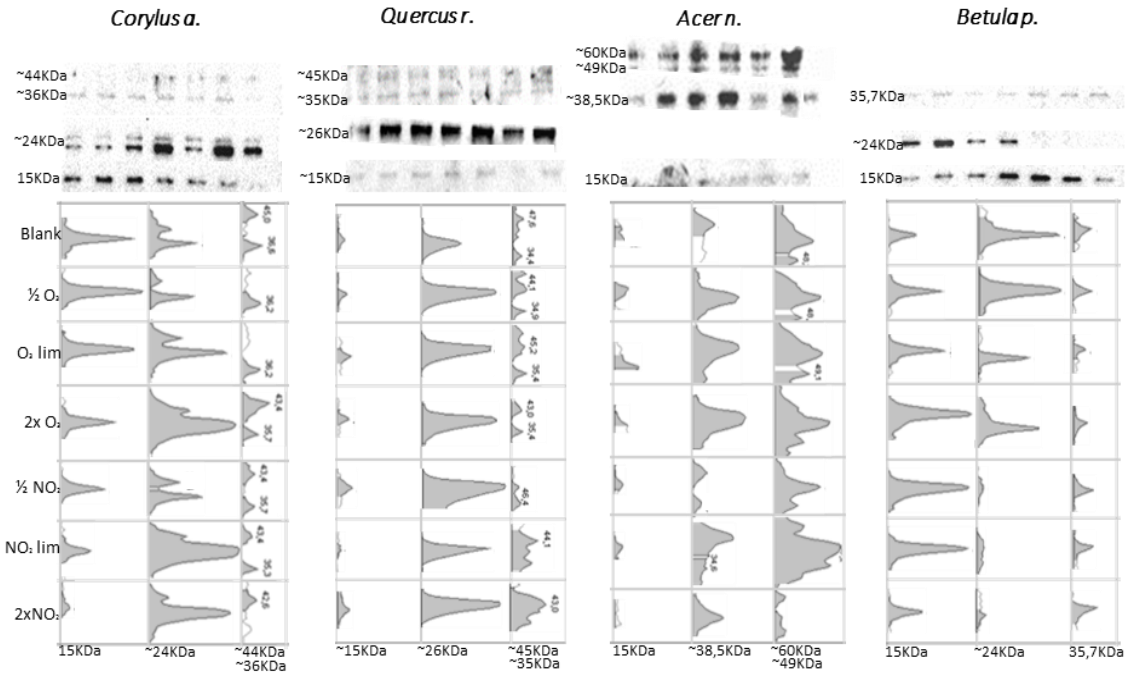

Figure 5. Immunodetection of SOD enzymes in pollen protein extracts (10 μg per lane) from *A. negundo, B. pendula, C. avellana and Q. robur* non exposed (B) and exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe. Top figures correspond to western blot probed with a customized anti-olive pollen Cu/Zn SOD Ab (sequence accession nº EU250769.1) and bottom graphics to differences in optical density of reactive bands.

316 The band of 15 KDa presented increase expression trend in pollen after exposure o O_3 for *A.*
317 *negundo*, *B. pendula* and *Q. robur*. Considering NO_2 the effect was dissimilar, inducing increased
318 expression for *B. pendula* and *Q. robur* pollen and decreased for *A. negundo* and *C. avellana*.
319 For the band around 35-36 KDa an increased expression trend after exposure to O_3 and NO_2 was
320 only observed for *A. negundo* pollen. In the case of *C. avellana* and *Q. robur*, NO_2 was the only gas
321 with visible influence (inducing decreased expression) but contrarily for *B. pendula* O_3 was the only
322 one altering the band expression.
323 Considering the band around 25 KDa, *Q. robur and C. avellana pollen presented increased expression*
324 after exposure to both gases, but for this last pollen species only the levels at the limit values and at
325 twice the limit value for vegetation protection induced the increase. For *B. pendula*, NO_2 induced a
326 great expression decrease while a negative influence of O_3 was only detected at the highest
327 concentration levels.

328 *3.4. Raman microspectroscopy*

329 Our results showed small changes in the pollen wall chemical structure after exposure to NO_2
330 and O_3 (Fig.6). Magnitude shifts varied between species and gas type, but they generally occurred at
331 the same wavenumber area. Visible changes can be observed at ≈ 1700-1800 cm^{-1} and at ≈ a triplet
332 region between 1300-1500 cm^{-1} (particular emphasis on one peak at ≈ 1440 cm^{-1}). For *A. negundo*
333 pollen, an additional change was observed at ≈ 1000-1200 cm^{-1} wavenumber. These changes occur in
334 the intensity of the spectra, and the flattening of some peaks into shoulders.

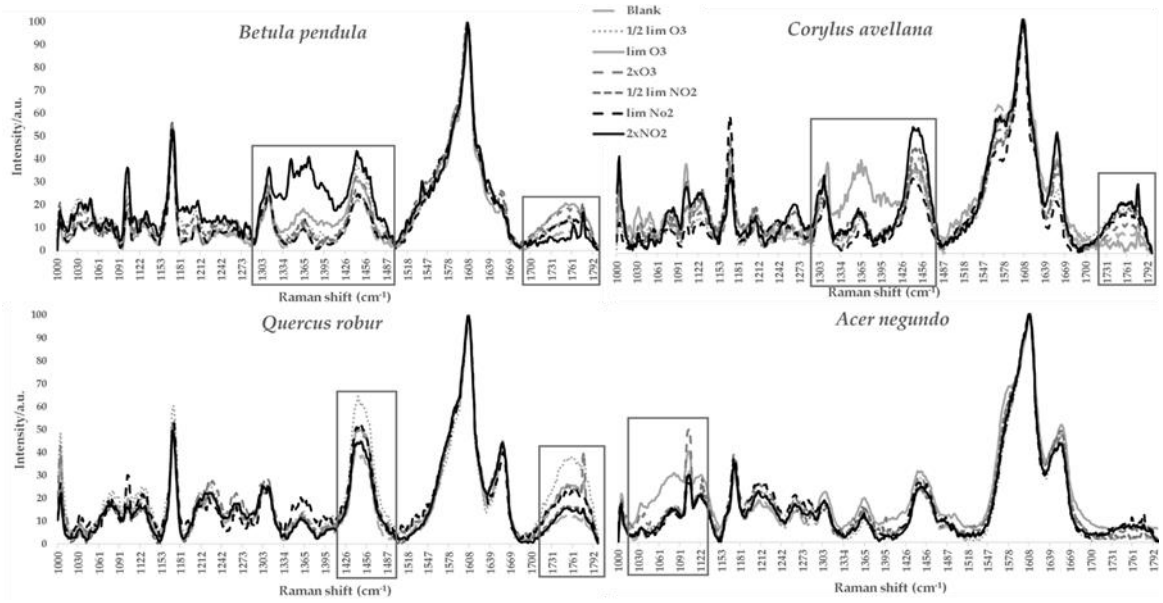

Figure 6. Average Raman spectra of *A. negundo, B. pendula, C. avellana* and *Q. robur* pollen, exposed for 6 h to O_3 and NO_2 at concentrations corresponding to half the limit (1/2), the limit (Lim) and the double limit (2xLim) values for the protection of vegetation according to the EU Directive 2008/50/EC on ambient air quality and cleaner air for Europe. The gray squares highlight the peaks where visible changes occur.

C. avellana pollen was the species showing the biggest changes in the spectra of exposed samples when compared with the blank one. Overall, the highest concentration of NO_2 have greatest impact on *B. pendula* pollen while for the other species O_3 seems to be the gas with the greatest effect.

4. Discussion

Many studies have focused on the influence of pollutants on pollen (reviewed in [26] but very few exist comparing different species at different pollutant levels under the same experimental design as in our study where the interactions between the air pollutants and pollen of 4 forest species were evaluated.

Overall, NO_2 seems to affect all species more than O_3. For this last gas, it was possible to observe that the greater effect occurred for pollen exposed to the limit value concentration, which could mean that for some species the tolerance threshold occurs at this concentration level.

It is, therefore, important to test more forest species and understand their resistance to atmospheric pollution regarding the vegetative development or pollen fertility, and how this can affect the crops and productivity of the forest. Air pollution exists, and it is crucial to understand its effects on vegetation to prepare the management and sustainability of the forest ecosystem.

Our pollen viability results showed that *A. negundo* pollen is the most resilient in terms of viability to O_3 and NO_2 comparing to the other species. Also, *C. avellana* pollen viability is more sensitive to O_3 than to NO_2 while Q. robur has equal sensibility to both pollutants, but the pollen viability of both species is affected even at level below the limit value for the protection of vegetation according to the EU Directive 2008/50/EC.

The gas influencing the most negatively the pollen viability was NO_2, particularly in those samples' exposure to the highest concentration.

The pollen viability is an important parameter to acknowledge pollen conditions and may indicate how deep the effects of pollution are on pollen, which has a direct effect on forests reproduction and crop.

A decrease in pollen viability after exposure to pollution, in situ or under laboratory controlled conditions, has been reported by many studies such as in Pasqualini et al. [29] for ragweed pollen after exposure to ozone, Leghari et al. [17] for in situ assessment on sweet cherry pollen grains exposed to vehicular exhaust pollution on a road site compared with a control sample apart from the polluted site. In this last study it was also reported changes on pollen grains size and quantity. Indeed, despite the different pollutant gases, concentrations and conditions of exposure tested and reported in the literature, pollen viability has been always consistently negatively affected by pollution. Although anemophilous trees, as the ones in our study, produce much more pollen to balance the loss due to air transport, the decrease in viability can attain levels not able to compensate the excess pollen production and therefore major loss in fruit production may occur in crop forests.

In our study, the effects of pollution on pollen total soluble protein (TSP) was contradictory when compared to the blank samples. In *A. negundo* and *Q. robur* species almost all samples exposed to the gas pollutants presented no change or a significant decrease in TSP content relative to the blank sample. The other species showed similar behavior, with most of the samples having equal or higher TSP content that the blank samples. In the literature increase and decrease in protein content has been observed in different species (REF). It has been argued that the effects are dependent on species and pollutant but comparisons among the different studies is always relative because distinct experimental conditions are present. In our study we enlighten this subject because the results from the same batches of pollen from four different pollen species subjected to two pollutant gases at distinct concentrations showed that some species react differently to gasses and the reaction is not always positively correlated with concentration. These findings support the different trends reported in the literature concerning pollen protein content and exposure to pollution.

In our study, the samples of pollen exposure to the pollutant gases, especially at the limit and two times limit concentration value for vegetation protection presented significant changes especially related to stress conditions, with increase in ROS production, NADPH oxidase activity and SOD expression.

The both gases tested have a similar influence on the production of ROS, inducing an increase, without any standing out within the species. *B. pendula* and *C. avellana* behave in a similar way where gases seem to influence less than in the case of *A. negundo* and *Q. robur*. Senechal et al. [26] referred that air pollutants may diffuse into plant cells leading to ROS production and consequently increasing stress, what it was observe overall in our study. Gases like ozone and nitrogen dioxide are oxidant pollutants, that create a stressful environmental to pollen, what can stimulate ROS production. ROS as superoxide ion, hydrogen peroxide, hydroxyl radical, have been reported as an important signaling toll in plants, being able to control major processes like growth, development and being essential in the response to abiotic and biotic environmental stimulus[27].

Comparing the ROS activity with the viability percentage it's possible to see that they are negatively related, when one increases the other decreases, since stress conditions affect negatively the pollen physiology and its expected that the effect it shows in the fertility.

Considering the NADPH oxidase enzymatic activity, in spite of being one of the main responsible for the depletion of ROS within the plant cells, was the parameter with the lowest change induced by the pollutant gases, with only the higher levels of concentration inducing increase in the activity and NO_2 being the most important.

In our study was observed an increase of the pollen SOD enzymatic activity after its exposure to the pollutant gases suggesting the existence of oxidative stress and showing that pollen oxidative defenses were activated by common air pollutants. Several evidences show that SOD, as well as other enzymes, are involved in stress tolerance in plants exposed to air pollutants serving as primary defense to avoid cell injury [37, 38] .

CuZn-SOD bands are reported also as major pollen allergens this meaning that the increase on the expression observed in our study may suggest an increase in pollen allergenic potency after daily exposure to air pollution [36]. As in all tests performed in our study differential band reactivity occurred depending on the pollen species, showing that some can be more resistant.

In this study it was analyzed the pollen of two species of the same family, Betulaceae family, namely *B. pendula* and *C. avellana* with both species showing a similar behavior, increase in pollen ROS and NADPH activity, as well as a decrease in viability. These species seems to be more sensitive to increasing NO_2 concentration levels, however a different behavior occur in O_3, were the most influence happen at the legal limit concentration, indicating that this concentration might be the breaking point for this type of pollen exposure, because after that, the stress indicators show a minor decrease. When compared with the other species analyzed it is possible to see different behaviors, especially for *Q. robur* pollen were lower levels of exposure, of both gases, seem to have more effects on the pollen. What confirms the already known idea, that each plant species may have a different susceptibility to the pollution levels in the atmosphere.

The pollen wall composition is another feature where changes can be found induced by the pollutant gases. As it happened in the other pollen features investigated in this study, A. negundo pollen seems to be less affected by the two pollutants analyzed. *B. pendula* and *C. avellana* show similar behavior, although as it happens in pollen viability, *C. avellana* is more sensitive to O_3 than to NO_2 while *B. pendula* is more affected with NO_2 exposure.

The wavenumbers were visible changes are observed can be assign to nucleic acids adenine and guanine (~1360 cm^{-1}) [39], amide III system (N– H, C–H deformation) (~1300 cm^{-1}), and deformation made of C-H_2 groups of aliphatic carbon chains [40], sporopollenin or lipids (1440 cm^{-1})[41]. The pollen wall has complex chemical composition, which are important to the structural maintenance of the pollen wall and protection of reproductive nuclei. DNA degradations, sporopollenin degradation as well as change in the carbon structures of the pollen wall can be determinant in the resilience and resistance of pollen grains to environmental pollution. Other authors also suggested changes in the same compounds, such as proteins, lipids, carbohydrates, sporopollenin after pollen exposure to O_3 and NO_2 [42, 43]. Ribeiro et al. [28], using FT-IR reported changes in the pollen wall of *Platanus* pollen, at the same components observed in our study after exposure to NO_2 and O_3 even at below the limit for the protection of human health according to the European Directive 2008/50 / EC on ambient air quality and cleaner air in Europe. The same was reported for Corylus pollen collected in areas with high levels of air pollution when compared to pollen from areas with lower levels of pollution [44].

It is important to highlight that, for each species, we are comparing spectra of pollen from the same batch, so the changes we are observing can occur when pollen is airborne exposed to peak pollution episodes. Additionally, the fact that each species shows different behaviors depending on the gas type and concentration but their effect occurs consistently at similar wavenumbers, meaning always at the same pollen wall constituents, points once again to species-specific resilience to pollution.

Air pollution is refereed by many authors, one of the most contributors for pollen increase allergies. Our focus for this study, was not the allergic reaction increase on the population but the

effect that may come to the pollen exposure during a day time to traffic related pollution as well as other sources, inside the legal limit value for human health protection in Europe and the half and the doble of this value, although in a forest zone many plant cultures are reunited and to the allergic population an increase on the allergenicity of the pollen do to air pollution, may occur, showing the importance of analyze this factor as well.

Also, more studies are need to understand the effect that may come to the plant at all levels, the seed to the fruit, as that may have some consequences in forests ecosystem, at production levels but as well as providing well-being system.

5. Conclusions

In this study pollen of four forest species were analyzed after exposure to NO_2 and O_3 gases, at three different concentrations. Our results suggest that changes in pollen viability, protein content and oxidative stress occurred after exposure, which may affect plant reproduction and growth.

For *Quercus robur*, *Acer negundo*, *Betula pendula* and *Corylus avellana* different behaviors were reported, with differential sensitivity related with ROS synthesis, NADPH oxidase activity as well as a in pollen viability and wall composition. Most species studied seem to be more affected by NO_2 gas exposure, being the samples exposure to the highest concentration, the ones more affected. As for ozone, there were less significant differences between samples, however a different behavior occur in O_3 expositions, were the most influence happen at the legal limit for vegetation protection in Europe, this seems to be the breaking point for this type of pollen exposure, because after that, the stress indicators of the pollen show a minor decrease.

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References

1. Dobbs, C., F.J. Escobedo, and W.C. Zipperer, *A framework for developing urban forest ecosystem services and goods indicators.* Landscape and Urban Planning, 2011. **99**(3-4): p. 196-206.
2. Ghazoul, J., *Forests: A Very Short Introduction*. Oxford University Press, 2015.
3. Rajoo, K.S., D.S. Karam, and M.Z. Abdullah, *The physiological and psychosocial effects of forest therapy: A systematic review.* Urban Forestry & Urban Greening, 2020. **54**: 126744
4. Krajter Ostoić, S., et al., *Urban forest research in the Mediterranean: A systematic review.* Urban Forestry & Urban Greening, 2018. **31**: p. 185-196.
5. Vujcic, M., et al., *Connection between urban green areas and visitors' physical and mental well-being.* Urban Forestry & Urban Greening, 2019. **40**: p. 299-307.

6. Berland, A., et al., *The role of trees in urban stormwater management.* Landscape Urban Planning, 2017. **162**: p. 167-177.
7. Xu, C., et al., *Can forest city construction affect urban air quality? The evidence from the Beijing-Tianjin-Hebei urban agglomeration of China.* Journal of Cleaner Production, 2020. **264**: 121607.
8. Nowak, D.J., et al., *Tree and forest effects on air quality and human health in the United States.* Environmental Pollution, 2014. **193**: p. 119-129.
9. Nowak, D.J. and D.E. Crane, *Carbon storage and sequestration by urban trees in the USA.* Environmental Pollution, 2002. **116**(3): p. 381-389.
10. Smith, W.H., *Forests, Crops, and Air Quality.* BioScience, 1990. **40**(10): p. 781-782.
11. Douglas, A.N.J., P.J. Irga, and F.R. Torpy, *Determining broad scale associations between air pollutants and urban forestry: A novel multifaceted methodological approach.* Environmental Pollution, 2019. **247**: p. 474-481.
12. Shiraiwa, M., K. Selzle, and U. Pöschl, *Hazardous components and health effects of atmospheric aerosol particles: reactive oxygen species, soot, polycyclic aromatic compounds and allergenic proteins.* Free Radical Research, 2012. **46**(8): p. 927-939.
13. Wielgosinski, G., *Pollutant Formation in Combustion Processes*. Advances in Chemical Engineering, 2012. p. 30.
14. Najjar, Y., *Gaseous Pollutants Formation and Their Harmful Effects on Health and Environment.* Ashdin Publishing Innovative Energy Policies, 2011. **1** (9)
15. Antonio, J., et al., *Plant Physiology and Biochemistry Oxidative stress in ryegrass growing under different air pollution levels and its likely effects on pollen allergenicity.* Plant Physiology and Biochemistry, 2019. **135**: p. 331-340.
16. Gill, S.S. and N. Tuteja, *Plant Physiology and Biochemistry Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants.* Plant Physiology et Biochemistry, 2010. **48**(12): p. 909-930.
17. Leghari, S.K., et al., *Response of sweet cherry (Prunus avium L.) pollen grains to vehicular exhaust pollution at Quetta*. Applied Ecology and Environmental Research, 2018. **16**(1981): p. 4387-4399.
18. Vander Mijnsbrugge, K., et al., *Does Drought Stress on Seedlings Have Longer Term Effects on Sapling Phenology, Reshooting, Growth and Plant Architecture in Quercus robur, Q. petraea and Their Morphological Intermediates?* Forests, 2019. **10**(11):1012
19. Sgrigna, G., et al., *Relationships between air particulate matter capture efficiency and leaf traits in twelve tree species from an Italian urban-industrial environment.* Science of The Total Environment, 2020. **718**:137310.
20. Hoshika, Y., et al., *Flux-Based Ozone Risk Assessment for a Plant Injury Index (PII) in Three European Cool-Temperate Deciduous Tree Species.* Forests, 2020. **11**(1):82
21. Yamaguchi, M., et al., *Evaluation of O3 Effects on Cumulative Photosynthetic CO2 Uptake in Seedlings of Four Japanese Deciduous Broad-Leaved Forest Tree Species Based on Stomatal O3 Uptake.* Forests, 2019. **10**(7): 556
22. Fröhlich-Nowoisky, J., et al., *Bioaerosols in the Earth system: Climate, health, and ecosystem interactions.* Atmospheric Research, 2016. **182**: p. 346-376.
23. Colls, J., *Air Pollution*. 2002, 2nd Edition: Spon Press, London.

24. Duque, L., et al., *Elemental characterization of the airborne pollen surface using Electron Probe Microanalysis (EPMA).* Atmospheric Environment, 2013. **75**: p. 296-302.
25. Oduber, F., et al., *Links between recent trends in airborne pollen concentration, meteorological parameters and air pollutants.* Agricultural and Forest Meteorology, 2019. **264**: p. 16-26.
26. Sénéchal, H., et al., *A Review of the Effects of Major Atmospheric Pollutants on Pollen Grains , Pollen Content , and Allergenicity.* Scientific World Journal, 2015.
27. Das, K. and A. Roychoudhury, *Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants.* Frontiers in Environmental Science, 2014. **2**: 53
28. Ribeiro, H., et al., *Science of the Total Environment Effect of O_3 and NO_2 atmospheric pollutants on Platanus x acerifolia pollen : Immunochemical and spectroscopic analysis.* Science of the Total Environment, 2017. **599-600**(2): p. 291-297.
29. Pasqualini, S., et al., *Ozone affects pollen viability and NAD(P)H oxidase release from Ambrosia artemisiifolia pollen.* Environmental Pollution, 2011. **159**(10): p. 2823-2830.
30. Sousa, R., et al., *In Vitro Exposure of Acer negundo Pollen to Atmospheric Levels of SO_2 and NO_2: effects on allergenicity and germination.* Environmental Science Technology, 2012. **46**(4): p. 2406-2412
31. Lu, S., et al., *Characterization of protein expression of Platanus pollen following exposure to gaseous pollutants and vehicle exhaust particles.* Aerobiologia, 2014. **30**: p. 281-291.
32. Silva, M., et al., *Effects of CO_2 on Acer negundo pollen fertility , protein content , allergenic properties , and carbohydrates.* Environmental Science Pollution Research, 2015. **22**: p. 6904-6911.
33. Ribeiro, H., et al., *Effect of O_3 and NO_2 atmospheric pollutants on Platanus x acerifolia pollen: Immunochemical and spectroscopic analysis.* Science of the Total Environment, 2017. **599-600**: p. 291-297.
34. Heslop-Harrison, J., Y. Heslop-Harrison, and K.R. Shivanna, *The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure*. Theoretical and Applied Genetics, 1984. **67**(4): p. 367-375.
35. Bradford, M.M., *A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding.* Analitycal Biochemistry, 1976. **72**: p. 248-254
36. Zafra, A., A.J. Castro, and J.D. Alche, *Identification of novel superoxide dismutase isoenzymes in the olive (Olea europaea L.) pollen.* BMC Plant Biology, 2018. **18**(1): 114.
37. Uka U. N., Hogarh J., Belford E. J. D., *Morpho-Anatomical and Biochemical Responses of Plants to Air Pollution.* International Journal of Modern Botany, 2017. **7**(1): 1-11
38. Rao, M.V. and P.S. Dubey, *Biochemical aspects (antioxidants) for development of tolerance in plants growing at different low levels of ambient air pollutants.* Environmental Pollution, 1990. **64**(1): p. 55-66.
39. Diehn, S., et al., *Combining Chemical Information From Grass Pollen in Multimodal Characterization.* Frontiers in Plant Science, 2020. **10**: 1785
40. Guedes, A., et al., *Pollen Raman spectra database: Application to the identification of airborne pollen.* Talanta, 2014. **119**: p. 473-478.

41. Ivleva, N.P., R. Niessner, and U. Panne, *Characterization and discrimination of pollen by Raman microscopy.* Analytical and Bioanalytical Chemistry, 2005. **381**(1): p. 261-267.
42. Kanter, U., et al., *Molecular and immunological characterization of ragweed (Ambrosia artemisiifolia L.) pollen after exposure of the plants to elevated ozone over a whole growing season.* PloS one, 2013. **8**(4): 61518
43. Zhao, F., et al., *Common ragweed (Ambrosia artemisiifolia L.): allergenicity and molecular characterization of pollen after plant exposure to elevated NO_2.* Plant, Cell and Environment, 2016. **39**(1): p. 147-64.
44. Depciuch, J., et al., *FTIR analysis of molecular composition changes in hazel pollen from unpolluted and urbanized areas.* Aerobiologia, 2017. **33**(1): p. 1-12.

5. General Discussion

Pollen has been suggested by many authors as a sensitive bioindicator of air quality, especially when data is needed in small scale, since atmospheric pollutants have significant impacts on airborne pollen traits, as demonstrated by the results attained in this dissertation. Pollen's vast presence and dispersion in urban areas becomes therefore a value in this scenario (Carneiro et al. 2011) and its automatic real-time monitoring has substantial influence in allergen exposure avoidance, which is the most effective measure to fight pollen allergies.

5.1. Pollen identification

Automatic pollen identification has been investigated by many authors for many years, and among the most successful are spectrophotometric techniques (Mondol et al. 2019; Zimmermann 2017). Raman spectroscopy has been used for pollen automatic identification, (Ivleva et al. 2005; Schulte et al. 2008; Zimmermann 2010). This technique is non-destructive and allows pollen classification based on the chemical characterization (chemical structure and molecular interactions) of its wall (Ivleva et al. 2005; Seifert et al. 2016; Mondol et al. 2019). Schulte et al.(2008) referred that most of the pollen species analyzed in his study presented chemical similarities, what is indicative of both phylogenetic relationship and mating behavior, but the differences showed promising to pollen discrimination.

Raman spectra peaks/wavenumbers can be attributed to a specific molecular bond vibration (both individual bonds, such as C-C, C=C, N-O, and groups of bonds). As a result of the different chemical composition between the pollen species, their Raman spectra are quite complex and variable, allowing to obtain a genus-specific and even species-specific chemical fingerprint of pollen (Kraaijeveld et al. 2015; Mondol et al. 2019) as confirmed by our results. The Raman spectra complexity and variability between distinct pollen types enables its identification and classification, by applying data analysis, to taxonomic levels that are many times impossible for humans under light microscopy (Kraaijeveld et al. 2015; Mondol et al. 2019).

In previous studies conducted with the aim to identify and classify different pollen species, the all spectra or a fraction of it within a pollen fingerprint region was used (Guedes et al. 2014; Seifert et al. 2016). Indeed, by visual inspection of the different spectra analyzed in our study is it possible to distinguish both common fingerprint areas

to all species as well as some characteristic fingerprint areas specific of only some pollen genus or species. As an example, when we compare between trees and grasses pollen spectra, in the band area ≈ 1300- 1460 cm^{-1}, the spectra of grasses show less define peaks but with high intensity, than the ones of tree pollen, where the peaks are more defined but with small intensity. The opposite tendency was observed in the band area ≈ 1500-1700 cm^{-1}, where tree-pollen spectra have a well-defined region with higher intensity that the spectra of grass species. So, profiting from these specific differences, in our study, article 1, we intended for the first time ever to test the use of the Raman spectra parameters (wavenumber position, the area under the peak and full width at half maximum of the band) into pollen identification and classification rather than the whole spectrum.

We analyzed 15 different pollen species, among trees, scrubs and weeds. In order to normalize and improve spectra quality, the spectra collected, in a small spectral range between 1000 and 1800 cm^{-1}, were pre-process (baseline, smoothing and normalize). Then to fulfill the objective of achieving pollen identification only by using the Raman parameter values, the spectra were deconvoluted using 18 wavenumbers position, the ones that best fit the deconvolution line. For the classification only the seven common band intervals for all pollen species were selected, distributed by three fingerprint regions (1000-1010; 1300-1460 and 1500-1700 cm-1).

The complexity of the Raman pollen spectra is so high that even with advanced pre-processing techniques, the signal-to-noise ratio is still considerable and therefore the need for the application of dimension-reduction techniques (e.g. principal components analysis) in combination with robust data mining methods are necessary to achieve successful results (Mondol et al. 2019; Zimmermann 2017). However, by performing the classification based only on the Raman parameters of the seven common bands using a supervised machine learning technique (Support Vector Machine – SVM) a more simplifying approach is applied and was sufficient to attain a successful discrimination between the pollen species.

In our study, the classification potential was evaluated in three dataset combinations, all 15 tested species together, only tree species and only grass species. This was done because we wanted to investigate if the classification procedure could be simplified by dividing it in a two-step classification; the first one more wider based on reduced number of fingerprint areas to discriminate trees from grasses and weeds and a second one more thorough, focusing on more fingerprint areas but with less number of species, which would allow to reduce the probability of a false-positive or negative classification.

The results obtained using all 15 species dataset showed the possibility for the proposed analytical methodology to be used in automatic pollen detection technique. Our results showed for the training step 100% classification accuracy (CA), precision and recall, that is, no species was wrongly classified. When we go onto to the test step, the classification is naturally more inaccurate. It was obtained a CA and recall of 90%, precision of 93.3% with 14 out of the 15 species being correctly identified, which are very positive results. For example, the pollen species *Corylus* and *Betula*, belonging to the Betulaceae family, are morphologically very similar and often difficult in identifying by optical microscope. Also, this similitude poses some challenges for the image processing-based pollen identification methods, only minimized by the desynchronized presence in the atmosphere of these pollen species (Sauvageat et al. 2020). In our study they are correctly identified, showing that the methodology proposed is an improving step to pollen identification.

However, when trying to perform a discrimination only between trees, the accuracy of the classification slightly worsens when compared to the test with all species (CA and recall of 77.8% and a precision of 66.7%). If we observe the spectra of the tree species, in the fingerprint area ≈1300-1460 cm^{-1}, the spectrum of *Quercus robur* pollen, now confused, presents much more similarity with grass species. Therefore, in the presence of all species, the spectra would end up distant from the other species of trees and closer to grass pollen spectra. When we remove the herbs from the group, the classification of this specific pollen specie worsens. Therefore, we needed all species to attain better results for trees and our intention of developing a two-step classification was not possible. Nonetheless, when we entered in the analysis only grass species, the classification attained was perfect, both in train and test steps, which is excellent and is one of the most striking points in this study. It is possible to distinguish with complete accuracy the five pollen species belonging to the same family, Poaceae, even though they are practically indistinguishable under the optical microscope, the traditional pollen identification method. Grass pollen are among the most common worldwide allergens, due to grasses widely distribution around the globe with a large number of species and extensive flowering season, that lasts around 4 to 5 months from March-July and September, with several annual peaks in airborne pollen concentration, which makes for months of suffering for allergic patients (Ribeiro and Abreu 2014). Furthermore, the pollen season of the most allergenic ones may be overlapped what may enhance the allergic individual reaction (García-Mozo 2017). So, it becomes clear the importance to identify the different airborne pollen contributors among the Poaceae family. Among the most allergic ones are genus such as *Lolium* spp., *Dactylis* spp., *Anthoxanthum* spp. and

Phleum spp. (García-Mozo 2017; Brennan et al. 2019), being a real challenge to exactly defined the traits of their airborne pollen seasons, beginning and ending, for the different genus (Brennan et al. 2019). Presently, the grass airborne pollen cannot be discriminated by genus or species due to morphological similarities. Features such as number of apertures, shape and texture are quite similar, posing great analytical challenges to image-processing algorithms (Ronneberger et al. 2002) but in our study the Raman parameters of only seven common band intervals from the full pollen spectrum were sufficient for the distinction between 5 different species of Poaceae.

Of course, more studies and a greater number of pollen species need to be tested, however in this repertoire of 15 species, we have the wide variety of species considered most allergenic. As such, in terms of public health, the possibility of identifying these species is already a great added value.

5.2. Pollutants-induced pollen changes

Along the last years, environmental pollution has been studied with the objective of understand the effects that occur in humans, animals and plants (Fernandez-Gonzalez et al. 2019; Oduber et al. 2019). Several air pollutants, namely NO_2 and O_3, have been tested in order to understand the effects they can induce in pollen. Most studies state that changes occur in several characteristics of the pollen grain, such as fertility, oxidative stress, and protein content (Lucas et al. 2019; Sénéchal et al. 2015). However, they do not always report a positive or negative influence of the factors mentioned, almost always is referred a variation depending on the pollen species, gases or mixture of gases and consequently the concentration to which the pollen was exposed while in the plant, in the air or in vitro.

In our study, *Acer negundo*, *Betula pendula, Corylus avellana* and *Quercus robur* pollen was analyzed after exposure to NO_2 and O_3 gases, at three different concentrations. Overall, it was observed an increase in ROS activity, NADPH oxidase activity as well as a decrease in pollen viability. Our results point out to most species are more affected by NO_2 exposure, particularly those samples exposed to the highest concentrations. As for ozone, there were less significant differences between samples.

5.2.1. Pollen fertility and protein content

Several studies have been conducted evaluating the influence of air pollutants on pollen fertility and all point to a reduction, being some pollen species more resilient than others, as showed in our study.

In our study pollen fertility was evaluated trough the assessment of pollen viability using a fluorochromatic reaction with fluorescein diacetate that enters the cell and reacts with esterases inducing fluorescence if the pollen grain is viable. Therefore, we are testing the influence of pollution on the enzymatic activity of pollen, that has been linked with its capacity to germinate.

In our study, a statistical significant reduction ($p<0.05$) on pollen viability was observed induced by O_3 and NO_2 but the percentage loss varied depending on pollen species, gas and concentration. For *A. negundo* and *B. pendula,* only the limit and the double legal limit value for NO_2 induced a significant reduction, while for *C. avellana* and *Q. robur* pollen that reduction happen at all samples exposed to NO_2. The same trend was observed by Sousa et al. (2012), that found a statistically significant drop in the germination rate of approximately 20%, for *Acer negundo* pollen, after exposure NO_2, when compared to non-exposed pollen samples and by Cuinica et al. (2014) for *Betula* and *Corylus* pollen after exposure to NO_2, CO, SO_2 and O_3.

Regarding the O_3 influence in pollen viability, for *A. negundo* and *B. pendula* pollen only the limit and the double legal limit concentrations induced a significant decrease. However, a significant negative effect was found on pollen viability for *C. avellana and Q. robu*r pollen. Pasqualini et al. (2011) also reported a decrease on the viability of ragweed pollen when expose to O_3.

The pollen viability is an important parameter to acknowledge pollen conditions and may indicate how deep the effects of pollution on pollen, which has a direct effect on forests reproduction and crop.

Regarding the total soluble protein (TSP) results showed distinct behavior. In the literature, both increase and decrease of TSP content are reported depending on pollen species and pollutants. However, it can be argued that these discordant trends can be a result of different experimental conditions (in vivo vs in vitro and whole plant vs only pollen exposure). Our study supports the different trends reported in the literature for TSP content as we analyzed four different pollen species exposed at the same experimental conditions. With the results, we conclude that different species react differently to gases and the reaction is not always positive correlated with the concentration.

5.2.2. Pollen oxidative stress

Air pollution is harmful to human health, gases like ozone and nitrogen dioxide are oxidant pollutants, that create a stressful environmental, what can stimulate ROS production in the plant cells, reactive oxygen species (superoxide ion, hydrogen

peroxide, hydroxyl radical), have been reported as an important signaling toll in plants, being able to control major processes like growth, development and being essential in the response to abiotic and biotic environmental stimulus (Leghari et al. 2018).

In order to evaluate the oxidative stress after pollen exposure to NO_2 and O_3 we observed three different oxidative markers. Reactive oxygen species (ROS), NADPH oxidase enzymatic activity and Superoxide dismutase (SOD) expression. The evaluation was made for all pollen samples in order to understand the changes that happen for different pollen species. Higher concentrations of gas exposure seem to induce changes especially related to stress conditions.

The behavior of all species points to an increase in ROS percentage after pollen exposure to the pollutants, although not all results are statistically significant when compared with the blank sample. The most affected species was *A. negundo* and the lowest was *C. avellana*.

It was observed that ROS activity and pollen viability were negatively related, so when one increases the other decrease, and therefore oxidative stress negatively affects the pollen physiology.

Considering the NADPH oxidase enzymatic activity, in spite of being one of the main responsible for the depletion of ROS within the plant cells, was the parameter with the lowest change induced by the pollutant gases, with only the higher levels of concentration inducing increase in the activity and NO_2 being the most important.

An in vivo study carried out by Lucas et al. (2019), on two different cities with different degrees of pollution support our results. In general, higher concentration of pollution induced more oxidative stress, with an increase in ROS and NADPH activity. The same authors also report a change in the plant physiological state particularly at photosynthetic efficiency and in the status of mechanisms involved in the metabolism related to oxidative stress. Other works referred changes at physiological level, this comes as an indication of what may be occurring in the plant exposed at air pollution and more studies are need to understand the effect in the plant at all levels, from the seed to the fruit, as that may have consequences in forests ecosystem, at production levels as well as providing well-being system.

Several evidences show that SOD, as well as other enzymes, are involved in stress tolerance in plants exposed to air pollutants, serving as primary defense to avoid cell injury (Rao and Dubey 1990; Uka U. N.1 2017).

In general, an increase in the reactivity was observed in our study for the samples after the exposure to pollutant, which shows that pollen oxidative defenses were activated by common air pollutants concentrations.

Additionally, some of CuZn-SOD bands are reported also as major pollen allergens meaning that the increase on the expression observed in our study may suggest an increase in pollen allergenic potency after daily exposure to air pollution (Zafra et al. 2018).

5.2.3. Chemical changes composition of the pollen wall

The pollen wall has a complex chemical composition consisting of proteins, lipids, carbohydrates and sporopollenin. Air pollutants influence pollen morphology and cause changes in pollen physiology, and in addition effect the chemical composition of the pollen wall (Kanter et al. 2013; Zhao et al. 2016).

With the intention of evaluate if pollutant gases (O_3 and NO_2) can induce changes on pollen wall, we use the Raman spectra of the studied species by comparing exposed and non-exposed samples.

Each pollen species shows different behavior but at similar wavenumbers, which suggest that specific constituents of the pollen wall are affected. Changes were particularly observed at bands assigned to nucleic acids (adenine and guanine) (~1360 cm^{-1} (Diehn et al. 2020), to amide III system (N– H, C–H deformation) (~1300 cm^{-1}) and to sporopollenin (~1440 cm^{-1}) (Ivleva et al. 2005). The pollen wall has a complex chemical composition; all these compounds can be important to the structural maintenance of pollen wall. DNA and sporopollenin degradation as well as change in the carbon structures of the pollen wall can be determinants in the resilience and resistance of pollen grains to environmental pollution. Changes in pollen wall proteins, lipids, carbohydrates, sporopollenin were also evidenced by other authors after pollen exposure to O_3 and NO_2 (Kanter et al. 2013; Zhao et al. 2016). Similar modifications have been reported for *Ambrosia* pollen that has been exposed to high concentrations of NO_2 or O_3 during growing season (Kanter et al. 2013; Zhao et al. 2016) and for *Corylus* pollen collected in areas with high levels of air pollution when compared to pollen from areas with lower levels of pollution (Depciuch et al. 2018).

As it happened with the other pollen features measured in our study*, A. negundo* pollen seems to be less affected by the two pollutants tested. *B. pendula* and *C. avellana* show similar behavior, although as it happens in pollen viability, *C. avellana* is more sensitive to O_3 than to NO_2 while *B. pendula* in this case seems to suffer more with NO_2

exposure. These differences may be due to diverse pollen mechanism when exposed to air pollution, as has been reported by other authors (Silva et al. 2015; Sousa et al. 2012).

For a more precise conclusion more studies need to be done in this area to achieve a better understanding of what happens to each species, and how it may influence their growth and other physiological mechanisms, how it can affect the cultures, actual and future ones.

6.General Conclusion and perspectives

6.1. Conclusion

Our study showed, for the first time, that it is possible to use only the Raman parameters to identify pollen species, with success. We were able to identify 14 out of 15 pollen species tested. Correct classification was achieved even for those pollen species that are difficult to distinguish at the optical microscope such as Poaceae species and between two species of the Betulaceae family such as *Betula pendula* and *Corylus avellana* usually distinguished by temporal discordance. By using the Raman parameters of the 7 common band intervals to all pollen species, we were able to reduce the volume of data necessary to successfully classify the pollen as well as the time of analysis and acquisition of each spectrum.

Regarding the effects of air pollutants on the pollen grain our results suggest the occurrence of significant differences in the fertility, protein content and oxidative stress after exposure, which may affect plant reproduction and growth. For *Q. robur*, *A. negundo*, *B. pendula* and *C. avellana* different behavior was observed, with differential sensitivity related with ROS activity, NADPH oxidase activity as well as in pollen viability. Most species analyzed were more affected by NO_2 exposure, particularly when exposed to the highest concentrations. For ozone, there were less significant differences between samples, however the most influence happened at the legal limit in Europe, which seems to be a breaking point because above this concentration the stress indicators measured in the pollen showed a lower reaction.

Comparative studies, such as the one reported by us, are important to understand what human actions may be causing in the environment, not only due to the response of plants to these actions, and the consequences that result, but also in order to minimize/ mitigate our impact. Pollen can be used as a sensitive bioindicator of the influence of air quality on plants.

Several measures have been taken with regard to achieving better air quality, and thanks to them some improvements have already been attained, however based on our study there is a need for even more demanding and restrictive measures, since changes in the pollen grain induced by the pollutant gases can occur even at atmospheric concentrations.

6.2. Perspectives

Our results contribute to improve the knowledge in the field of pollen automatic identification and the effects of air pollutants on pollen traits. Although there's still goals possible to achieve, that would benefit the environmental and public health.

Pollen identification using Raman parameters is a new methodology that could be incorporated as a component of a real-time automatic pollen detection systems. Due to its novelty, more multidisciplinary studies are need in order to optimize a robust real-time system. More spectra per species and a great number of species need to be analyzed, to cover all possible pollen present in the air. It will be also important testing the minimal acquisition time needed for spectra collection that allow still to have a precise classification.

In the scope of pollution influence there is the need in future studies to test more pollen species, to ascertain species the sensitivity and tolerance, and to evaluate metabolomic and genomic changes induced. This would give more insights to understand and describe in greater detail what happens at the intracellular level.

7. References

Ayenan MAT, Danquah A, Ampomah-Dwamena C, Hanson P, Asante IK, Danquah EY (2020) Optimizing ollencounter for high throughput phenotyping of pollen quality in tomatoes. *MethodsX* 7, art nº 100977.

Ba M, Zimmermann B, Kohler A (2015) A multiscale vibrational spectroscopic approach for identification and biochemical characterization of pollen. *PLoS ONE* **10** (9): e0137899.

Baksay S, Pornon A, Mariette J, Christophe A, Escaravage N (2020) Experimental quantification of pollen with DNA metabarcoding using ITS1 and trnL. *Scientific Reports,* **10** (1)**,** art nº 4202*.*

Bell KL, Vere ND, Keller A, Richardson RT, Gous A, Burgess KS, Brosi BJ (2016) Pollen DNA barcoding: current applications and future. *Genome*, **59**: 629–640.

Bist A, Pandit T, Bhatnagar AK, Singh AB (2004) Variability in protein content of pollen of Castor bean (*Ricinus communis*) before and after exposure to the air pollutants SO and NO. *Grana* **43** (2):94-100.

Blackmore S, Wortley AH, Skvarla JJ, Rowley JR (2007) Pollen wall development in flowering plants. *New Phytologist* **174** (3):483-498.

Bosch-Cano F, Bernard N, Sudre B, Gillet F, Thibaudon M, Richard H, Badot P-M, Ruffaldi P (2011) Human exposure to allergenic pollens: A comparison between urban and rural areas. *Environmental research* **111**:619-625.

Brennan GL, Potter C, de Vere N, Griffith GW, Skjoth CA, Osborne NJ, Wheeler BW, McInnes RN, Clewlow Y, Barber A, Hanlon HM, Hegarty M, Jones L, Kurganskiy A, Rowney FM, Armitage C, Adams-Groom B, Ford CR, Petch GM, Poller GENC, Creer S (2019) Temperate airborne grass pollen defined by spatio-temporal shifts in community composition. *Nature Ecology Evolution* **3**(5):750-754.

Carneiro MFH, Ribeiro FQ, Fernandes-filho FN, Lobo DJA, Jr FB, Rhoden CR, Mauad T, Saldiva PHN, Carvalho-oliveira R (2011) Pollen abortion rates, nitrogen dioxide by passive diffusive tubes and bioaccumulation in tree barks are effective in the characterization of air pollution. *Environmental and Experimental Botany* **72** (2):272-277.

Chen Y, Han L, Zhou Y, Yang L, Guo YS (2020) *Artemisia* Pollen Extracts Exposed to Diesel Exhaust Enhance Airway Inflammation and Immunological Imbalance in Asthmatic Mice Model. *International Archives of Allergy and Immunology*, **181** (5):342-352.

Chichiriccò G, Picozzi P (2007) Reversible Inhibition of the Pollen Germination and the Stigma Penetration in Crocus vernus ssp. vernus (Iridaceae) Following Fumigations with NO_2, CO, and O_3 Gases. *Plant biology* **9**:730-735.

Cuinica LG, Abreu I, Silva JCGE (2014) In vitro exposure of *Ostrya carpinifolia* and *Carpinus betulus* pollen to atmospheric levels of CO, O_3 and SO_2. *Environmental Science Pollution Research* **21**(3):2256-2262.

D'Amato G (2011) Effects of climatic changes and urban air pollution on the rising trends of respiratory allergy and asthma. *Multidisciplinary respiratory medicine* **6** (1):28-37.

Dafni A, Firmage D (2000) Pollen and Pollination. *Plant Systematics and Evolution* **222**:113-132.

Das K, Roychoudhury A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science* **2**: 53. doi: 10.3389/fenvs.2014.00053

Depciuch J, Kasprzyk I, Drzymała E (2018) Identification of birch pollen species using FTIR spectroscopy. *Aerobiologia* **34** (4):525-538.

Diehn S, Zimmermann B, Tafintseva V, Seifert S, Bagcioglu M, Ohlson M, Weidner S, Fjellheim S, Kohler A, Kneipp J (2020) Combining Chemical Information From Grass Pollen in Multimodal Characterization. *Frontiers in Plant Science* **10**, Artº 1788

Doughty DC, Hill SC (2020) Raman spectra of atmospheric particles measured in Maryland , USA over 22 . 5 h using an automated aerosol Raman spectrometer. *Journal of Quantitative Spectroscopy and Radiative Transfer* **244**, Artº106839.

Du Bay DT, Murdy WH (1983) The Impact of Sulfur Dioxide on Plant Sexual Reproduction: In Vivo and In Vitro Effects Compared. *Journal of Environmental Quality* **12** (1):147-149.

Duque L, Guimarães F, Ribeiro H, Sousa R, Abreu I (2013) Elemental characterization of the airborne pollen surface using Electron Probe Microanalysis (EPMA). *Atmospheric Environment* **75**:296-302.

Duque L, Noronha F, Ribeiro H, Guimar F, Abreu I (2015) Characterisation of particulate matter on airborne pollen grains. *Environmental pollution* **206**: 7-16.

Fernandez-Gonzalez M, Ribeiro H, Pereira JRS, Rodriguez-Rajo FJ, Abreu I (2019) Assessment of the potential real pollen related allergenic load on the atmosphere of Porto city. Science of The Total Environment, **668**: 333-341.

Fernandez-Gonzalez, M, Ribeiro H, Piña-rey A, Abreu I, Rodriguez-Rajo FJ (2020) Integrating Phenological , Aerobiological and Weather Data to Study the Local and Regional Flowering Dynamics of Four Grapevine Cultivars. *Agronomy*, **10**(2): art nº185.

Forde E, Gallagher M, Walker M, Foot V, Attwood A, Granger G, Sarda-Estève R, Stanley W, Kaye P, Topping D (2019) Intercomparison of multiple UV-LIF spectrometers using the aerosol challenge simulator. *Atmosphere* **10** (12):1-29.

France I, Duller AWG, Duller GAT, Lamb HF (2000) A new approach to automated pollen analysis. *Quaternary Science Reviews* **19**(6): 537-546.

Fuertes E, Markevych I, Berg AV, Bauer C-p, Berdel D, Koletzko S, Sugiri D, Heinrich J (2014) Greenness and allergies : evidence of differential associations in two areas in Germany. *Journal Epidemiological Community Health* **68**:787-790.

Fukuhara M, Fujiwara K, Maruyama Y, Itoh H (2019) Analytica Chimica Acta Feature visualization of Raman spectrum analysis with deep convolutional neural network. *Analytica Chimica Acta* **1087**:11-19.

García-Mozo H (2017) Poaceae pollen as the leading aeroallergen worldwide: A review. *Allergy* **72** (12):1849-1858.

Gillespie C, Stabler D, Tallentire E, Goumenaki E, Barnes J (2015) Exposure to environmentally-relevant levels of ozone negatively influence pollen and fruit development. *Environmental Pollution* **206**:494-501.

Guedes A, Ribeiro H, Fernández-González M, Aira MJ, Abreu I (2014) Pollen Raman spectra database: Application to the identification of airborne pollen. *Talanta* **119**:473-478.

Guedes A, Ribeiro N, Ribeiro H, Oliveira M, Noronha F, Abreu I (2009) Comparison between urban and rural pollen of *Chenopodium alba* and characterization of adhered pollutant aerosol particles*. Journal of Aerosol Science* **40**(1): 81-86.

Hong Q, Zhou S, Zhao H, Peng J, Li Y, Shang Y, Wu M, Zhang W, Lu S, Li S, Yu S, Wang W, Wang Q (2018) Allergenicity of recombinant *Humulus japonicus* pollen allergen 1 after combined exposure to ozone and nitrogen dioxide. *Environmental Pollution* **234**:707-715.

Ivleva NP, Niessner R, Panne U (2005) Characterization and discrimination of pollen by Raman microscopy. *Analytical and Bioanalytical Chemistry* **381**(1):261-267.

Kanter U, Heller W, Durner J, Winkler JB, Engel M, Behrendt H, Holzinger A, Braun P, Hauser M, Ferreira F, Mayer K, Pfeifer M, Ernst D (2013) Molecular and immunological characterization of ragweed (*Ambrosia artemisiifolia* L.) pollen after exposure of the plants to elevated ozone over a whole growing season. *PloS one* **8**(4):e61518

Kraaijeveld K, de Weger LA, Ventayol García M, Buermans H, Frank J, Hiemstra PS, den Dunnen JT (2015) Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. *Molecular Ecology Resources* **15**(1): 8-16.

Leghari SK, Saeed S, Asrar M, Ahmed A, Tariq I, Marri AA, Sadiq N, Baloch A, Latif A, Shawani NA (2018) Response of Sweet Cherry (*Prunus Avium* L.) Pollen Grains

to Vehicular Exhaust Pollution at Quetta, Balochistan, Pakistan. *Applied Ecology and Environmental Research* **16**(4): 4387-4399.

López-vizcaíno R, Yustres A, Sáez C, Cañizares P, Asensio L, Navarro V, Rodrigo MA (2019) Techno-economic analysis of the scale-up process of electrochemically-assisted soil remediation. J*ournal of Environmental Management* **231**: 570-575.

Lucas JA, Gutierrez-Albanchez E, Alfaya T, Feo-Brito F, Gutierrez-Manero FJ (2019) Oxidative stress in ryegrass growing under different air pollution levels and its likely effects on pollen allergenicity*. Plant Physiology and Biochemistry* **135**: 331-340.

Majd A, Chehregani A, Moin M, Gholami M, Kohno S, Nabe T, Shariatzade MA (2004) The Effects of Air Pollution on Structures, Proteins and Allergenicity of Pollen Grains. *Aerobiologia* **20**(2):111-118.

Manikis GC, Marias K, Alissandrakis E, Perrotto L, Savvidaki E, Vidakis N. (2019). Pollen Grain Classification using Geometrical and Textural Features. 2019 *IEEE International Conference on Imaging Systems and Techniques (IST)* Artº 19410551.

Marcos JV, Nava R, Cristóbal G, Redondo R, Escalante-Ramírez B, Bueno G, Déniz Ó, González-Porto A, Pardo C, Chung F, Rodríguez T (2015) Automated pollen identification using microscopic imaging and texture analysis. *Micron* **68**: 36-46.

Medek DE, Simunovic M, Erbas B, Katelaris CH, Lampugnani ER, Huete A, Beggs PJ, Davies JM (2019) Enabling self-management of pollen allergies : a pre-season questionnaire evaluating the perceived benefit of providing local pollen information. *Aerobiologia* **35**: 777-782.

Menad H, Ben-Naoum F, Amine A (2019) A Thresholding Approach for Pollen Detection in Images Based on Simulated Annealing Algorithm. *International Journal of Agricultural and Environmental Information Systems* **10**: 18-36.

Mondol AS, Patel MD, Rüger J, Stiebing C, Kleiber A, Henkel T, Popp J, Schie IW (2019) Application of high-throughput screening raman spectroscopy (HTS-RS) for label-free identification and molecular characterization of Pollen. *Sensors* **19** (20): 4428-4442.

Mularczyk-Oliwa M, Bombalska A, Kaliszewski M, Włodarski M, Kopczyński K, Kwaśny M, Szpakowska M, Trafny EA (2012) Comparison of fluorescence spectroscopy and FTIR in differentiation of plant pollens. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, **97**:246-254.

Muthreich F, Zimmermann B, Birks HJB, Vila-viçosa CM, Seddon AWR (2020) Chemical variations in *Quercus* pollen as a tool for taxonomic identification : Implications for long-term ecological and biogeographical research. *Journal of Biogeography*, 47(6): 1-12.

Oduber F, Calvo AI, Blanco-alegre C, Castro A, Vega-maray AM, Valencia-barrera RM (2019) Agricultural and Forest Meteorology Links between recent trends in airborne pollen concentration , meteorological parameters and air pollutants. *Agricultural and Forest Meteorology* **264**:16-26.

Okuyama Y, Matsumoto K, Okochi H, Igawa M (2007) Adsorption of air pollutants on the grain surface of Japanese cedar pollen. *Atmospheric Environment* **41**:253–260.

Okwuashi O, Ndehedehe CE (2020) Deep support vector machine for hyperspectral image classification. *Pattern Recognition* **103:**107298.

Oteros J, Pusch G, Weichenmeier I, Heimann U, Möller R, Röseler S, Traidl-Hoffmann C, Schmidt-Weber C, Buters JTM (2015) Automatic and Online Pollen Monitoring. International *Archives of Allergy and Immunology* **167**(3):158-166.

Pacini E, Franchi GG (2020) Pollen biodiversity - Why are pollen grains different despite having the same function? A review. *Botanical Journal Linnean Society*, **193**(2):141-164.

Parui S, Mondal AK, Mandal S (1998) Protein content and patient skin test sensitivity of the pollen of *Argemone mexicana* on exposure to SO_2. *Grana* **37**(2):121-124.

Pasqualini S, Tedeschini E, Frenguelli G, Wopfner N, Ferreira F, Amato GD, Ederli L (2011) Ozone affects pollen viability and NAD(P)H oxidase release from *Ambrosia artemisiifolia* pollen. *Environmental Pollution* **159**(10): 2823-2830.

Pawankar R, Canonica G. W, Holgate S. T and Lockey R. F, “White Book on Allergy 2011-2012 Executive Summary,” World Allergy Organization, USA, 2012.

Rahl M (2008) Microscopic Identification and Purity Determination of Pollen Grains. *Methods Molecular Medicine* **138** :263-269.

Ranzato M, Taylor PE, House JM, Flagan RC, LeCun Y, Perona P (2007) Automatic recognition of biological particles in microscopic images. *Pattern Recognition Letters* **28**(1): 31-39.

Rao MV, Dubey PS (1990) Biochemical aspects (antioxidants) for development of tolerance in plants growing at different low levels of ambient air pollutants. *Environmental Pollution* **64**(1): 55-66.

Reinmuth-Selzle K, Kampf CJ, Lucas K, Lang-Yona N, Fröhlich-Nowoisky J, Shiraiwa M, Lakey PSJ, Lai S, Liu F, Kunert AT, Ziegler K, Shen F, Sgarbanti R, Weber B, Bellinghausen I, Saloga J, Weller MG, Duschl A, Schuppan D, Pöschl U (2017) Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjuvants. *Environmental Science and Technology* **51**(8): 4119-4141.

Reis CIC, Coimbra-dores MJ, Rebelo MT, Reis CIC, Coimbra-dores MJ, Rebelo MT, Jo M, Rebelo MT (2018) Palynological analysis of soil in Portugal: potential for forensic science Palynological analysis of soil in Portugal : potential for forensic science. *Palynology* **43**(4): 585-595.

Rezanejad F (2009) Air pollution effects on structure, proteins and flavonoids in pollen grains of *Thuja orientalis* L.(Cupressaceae). *Grana* **48**: 205-213.

Ribeiro H, Abreu I (2014) A 10-year survey of allergenic airborne pollen in the city of Porto (Portugal). *Aerobiologia* **30**(3):333-344. doi:10.1007/s10453-014-9331-9

Ribeiro H, Costa C, Abreu I, Esteves da Silva JCG (2017) Effect of O_3 and NO_2 atmospheric pollutants on *Platanus x acerifolia* pollen: Immunochemical and spectroscopic analysis. *Science of Total Environmental* **599-600**:291-297.

Rogerieux F, Godfrin D, Sénéchal H, Motta A, Marliere M, Peltre G, Lacroix G (2007) Modifications of *Phleum pratense* Grass Pollen Allergens following Artificial Exposure to Gaseous Air Pollutants (O_3, NO_2, SO_2). *International archives of allergy and immunology* **143**:127-134.

Rojo J, Núñez A, Lara B, Sánchez-Parra B, Moreno DA, Pérez-Badia R (2019) Comprehensive analysis of different adhesives in aerobiological sampling using optical microscopy and high-throughput DNA sequencing. *Journal of Environmental Management* **240**: 441-450.

Ronneberger O, Schultz E, Burkhardt H (2002) Automated pollen recognition using 3D volume images from fluorescence microscopy. *Aerobiologia* **18**(2):107-115.

Ruske S, Topping DO, Foot VE, Morse AP, Gallagher MW (2018) Machine learning for improved data analysis of biological aerosol using the WIBS. *Atmospheric Measurement Techniques* **11**(11): 6203-6230.

Šantl-temkiv T, Sikoparija B, Maki T, Carotenuto F, Amato P, Yao M, Morris CE, Schnell R, Pöhlker C, Demott PJ, Hill TCJ, Alex J, Sikoparija B, Maki T, Carotenuto F, Amato P, Yao M, Morris CE, Schnell R, Jaenicke R, Demott PJ, Hill TCJ, Huffman JA, Demott PJ, Hill TCJ, Huffman JA (2020) Bioaerosol field measurements :

Challenges and perspectives in outdoor studies. *Aerosol Science and Technology* **54**(5): 520-546.

Sauvageat E, Zeder Y, Auderset K, Calpini B, Clot B, Crouzy B (2020) Real-time pollen monitoring using digital holography. *Atmospheric Measurement Techniques* **13**(3):1539-1550.

Schiavoni G, Amato GD, Afferni C (2017) The dangerous liaison between pollens and pollution in respiratory allergy. *Annals of Allergy, Asthma & Immunology* **118**(3):269-275.

Schopf JW, Calça CP, Garcia AK, Kudryavtsev AB, Souza PA, Félix CM, Fairchild TR (2016) In situ confocal laser scanning microscopy and Raman spectroscopy of bisaccate pollen from the Irati Subgroup (Permian, Paraná Basin, Brazil): Comparison with acid-macerated specimens. *Review of Palaeobotany and Palynology* **233**:169-175.

Schulte F, Lingott J, Panne U, Kneipp J (2008) Chemical characterization and classification of pollen. *Analytical Chemistry* **80**(24):9551-9556.

Seddon AWR, Festi D, Robson TM (2019) and challenges using proxy-system modelling. *Photochemical & Photobiological Sciences.* **18**(2),275-294.

Sedghy F, Sankian M, Moghadam M, Ghasemi Z (2017) Impact of traffic-related air pollution on the expression of *Platanus orientalis* pollen allergens*. International Journal of Biometeorology*. 61(1): 1-9.

Seifert S, Merk V, Kneipp J (2016) Identification of aqueous pollen extracts using surface enhanced Raman scattering (SERS) and pattern recognition methods. *Journal of Biophotonic*, **189** (1):181-189.

Sénéchal H, Visez N, Charpin D, Shahali Y, Peltre G, Biolley J-p, Lhuissier F, Couderc R, Yamada O, Malrat-domenge A, Pham-thi N, Poncet P, Sutra J-p (2015) A Review of the Effects of Major Atmospheric Pollutants on Pollen Grains , Pollen Content, and Allergenicity. *The Scientific World Journal* 2015: 1-29..

Sgrigna G, Baldacchini C, Dreveck S, Cheng Z, Calfapietra C (2020) Relationships between air particulate matter capture efficiency and leaf traits in twelve tree species from an Italian urban-industrial environment. *Science of The Total Environment* **718**, Artº137310.

Shiraiwa M, Selzle K, Pöschl U (2012) Hazardous components and health effects of atmospheric aerosol particles: reactive oxygen species, soot, polycyclic aromatic compounds and allergenic proteins. *Free radical research* **46**(8):927-939.

Silva M, Ribeiro H, Abreu I, Cruz A (2015) Effects of CO_2 on *Acer negundo* pollen fertility, protein content, allergenic properties, and carbohydrates. *Environmental Science Pollution Research* **22:** 6904-6911.

Soares C, Pereira R, Fidalgo F (2018) Metal-Based Nanomaterials and Oxidative Stress in Plants: Current Aspects and Overview. *Phytotoxicity of Nanoparticles*, **8**:197-227.

Sousa R, Duque L, Duarte AJ, Gomes CR, Ribeiro H, Cruz A, Esteves da Silva JC, Abreu I (2012) In vitro exposure of *Acer negundo* pollen to atmospheric levels of SO_2 and NO_2: effects on allergenicity and germination. *Environmental Science Technology* **46**(4): 2406-2412.

Speranza A, Crinelli R, Scoccianti V, Geitmann A (2012) Reactive oxygen species are involved in pollen tube initiation in kiwifruit. *Plant Biology* **14**(1):64-76.

Stanley RG, Linskens HF (1974) Pollen: biology, biochemistry, management. Springer-Verlag, Berlin; New York. pp 310.

Uka U N, Hogarh J, Belford E J D(2017) Morpho-Anatomical and Biochemical Responses of Plants to Air Pollution. *International Journal of Modern Botany* **7**(1): 1-11.

Wang C, Pan YL, Hill SC, Redding B (2015) Photophoretic trapping-Raman spectroscopy for single pollens and fungal spores trapped in air. *Journal of Quantitative Spectroscopy and Radiative Transfer* **153**:4-12.

Weiss R, Palatinszky M, Wagner M, Niessner R, Elsner M, Seidel M, Ivleva NP (2019) Surface-enhanced Raman spectroscopy of microorganisms: Limitations and applicability on the single-cell level. *Analyst* **144**(3): 943-953.

Xu Xl, Zheng Yz, Chen Xc, Zhu Fl, Miao Xq (2018) Identification of cattail pollen, pine pollen and bee pollen by Fourier transform infrared spectroscopy and two-dimensional correlation infrared spectroscopy. *Journal of Molecular Structure* **1167**(15):78-81.

Zafra A, Castro AJ, Alche JD (2018) Identification of novel superoxide dismutase isoenzymes in the olive (*Olea europaea* L.) pollen. *BMC Plant Biology* **18**(1),114.

Zhang M, Klimach T, Ma N, Tobias K, Poehlker C, Wang Z, Kuhn U, Scheck N, Pöschl U, Su H, Cheng Y, Zhang M, Klimach T, Ma N, Könemann T, Pöhlker C (2019) Size-resolved Single-particle Fluorescence Spectrometer for Real-time Analysis of Bioaerosols: Laboratory Evaluation and Atmospheric Measurements. *Environmental Science Technology* **53**(22):13257–13264

Zhao F, Elkelish A, Durner J, Lindermayr C, Winkler JB, Ruëff F, Behrendt H, Traidl-Hoffmann C, Holzinger A, Kofler W, Braun P, von Toerne C, Hauck SM, Ernst D, Frank U (2016) Common ragweed (*Ambrosia artemisiifolia* L.): allergenicity and molecular characterization of pollen after plant exposure to elevated NO_2. *Plant, cell & environment* **39**(1):147-164.

Zimmermann B (2010) Characterization of pollen by vibrational spectroscopy. *Applied Spectroscopy* **64**(12):1364-1373.

Zimmermann B (2017) Chemical characterization and identification of Pinaceae pollen by infrared microspectroscopy. *Planta* **247**(1):171-180.

Zimmermann B, Kohler A (2014) Infrared spectroscopy of pollen identifies plant species and genus as well as environmental conditions. *PLoS ONE* **9**(4), e95417.