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Effects of Environmental Stress on the Pollen Viability of Ornamental Tree-Species in the City of Granada (South-Eastern Spain)

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Abstract: Atmospheric conditions, as well as pollutants, can induce changes in the viability and germinability of the pollen grain. This process frequently occurs in cities due to the high rate of air pollution that can alter the quality of pollen, affecting its biological functions. In this work, the effect of different environmental stress factors, mainly UV-B radiation and polluting gases (CO, NO_2 and SO_2), on the viability and maturity of the pollen of four ornamental tree-species present in the green infrastructure of Granada, namely *Acer negundo*, *Carpinus betulus*, *Olea europaea* and *Cupressus* spp. is analyzed. Differential staining techniques were used with fresh pollen collected in areas with different exposure to environmental stress to detect intact cell membranes (Trypan blue) and the state of maturity (Pyrogallol red). It was observed that the species from sectors more exposed to environmental stress registered a low viability and were affected by factors such as UV-B radiation and atmospheric pollutants. On the contrary, the pollen from tree species growing in peri-urban forests presented a higher rate of viability and less effect of pollutant factors. Differences were also observed according to the species/genus and according to the sampling area. This modification in the morphological and/or organic composition of the pollen wall may cause a loss of quality in the reproductive processes of plants, and it may be bioindicator of the process of progressive degradation that plant species can experience in urban environments under conditions of environmental stress, and prevent the impacts that can affect other species.

Keywords: environmental stress; pollen viability; pollen maturity; ultraviolet-B radiation; air pollution

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1. Introduction

Variations in environmental conditions can impose important restrictions on the growth and development of plants and, therefore, cause stress situations on them. In this sense, the concept of "stress" implies the presence of a factor external to the plant, caused by the changing environment, which exerts a negative influence on its growth and optimal development [1]. In urban environments, urban trees can suffer a greater degree of stress as some of the environmental factors become more acute and they are also subjected to different maintenance actions carried out by man [2,3]. Most of the time, it is difficult to eliminate the stress factor, so the plants generate a response to it, such as the modification of the photosynthetic function [4], increasing their vulnerability to biotic agents in the form of pests and diseases, or altering the biological quality of some of its essential structures [5], such as the male gametophytes (pollen grains) essential for the reproductive process [6].

There is evidence that under certain stressful environmental conditions, such as those recorded in urban ecosystems, pollen grains can modify the organic composition of their cover, and that consequently, an alteration of pollen fertility and viability occurs, with a direct impact on the reproductive phenology of the species [7]. The impacts are so variable and they will depend both on the environmental conditions, on the concentrations of pollutants and on the morphological characteristics of the different pollen types. Some studies have shown that germination and soluble proteins in the exine of pollen grains of some species of the Betulaceae family (*Betula pendula*, *Ostria carpinifolia* and *Carpinus betulus*) are affected by NO_2 exposure in vitro even below the current atmospheric hour-limit value acceptable for human health protection [8]. The high concentrations of NO_2 and O_3 in polluted areas can also promote the nitration of proteins in the pollen wall, forming long-lived reactive oxygen intermediates (ROIs) and protein dimers, that cause loss of pollen viability [9]. In species such as *Ambrosia artemisiifolia*, the exposure of pollen to atmospheric pollutants has increased the expression of some proteins, causing a degradation of the pollen structure and significantly reducing its viability [10]. A similar effect occurs when pollen grains from *Quercus*, *Festuca* and *Ulmus* are subjected to low CO, NO_2 and SO_2 concentrations, where after exposure a change is observed in the water-soluble proteins and molecular weight [11]. Oak pollen exposed to 0.5 ppm NO_2 and SO_2 for 4 h showed morphological damage and loss of viability, detected using Trypan blue stain [12]. The light, partial or no acetocarmine staining of the morphologically altered pollen grains of several legume species in areas affected by fluoride pollution in Iran, in relation to the clear staining of the scarcely altered pollen grains from non-contaminated areas, has revealed the essential role of the pollen grain as a biomonitor of air pollution [13].

However, there are studies that suggest that some species of plants try to respond adequately to the presence of atmospheric pollutants, increasing the content of enzymes and flavonoids in their pollen grains as a protection mechanism, as has been observed in *Thuja orientalis* [14]. Some of the main tree species in the Mediterranean region, such as those of the *Quercus*, *Pinus* and *Olea* genera, have been shown to have a high tolerance to atmospheric O_3 concentrations due to the opening and closing control mechanism stomata [15], which has not affected either the production or the viability of their pollen grains [16]. In studies in which the pollen viability of four forest species has been evaluated: *Acer negundo*, *Corylus avellana*, *Quercus robur* and *Betula pendula*, after exposure to the same concentrations of O_3 and NO_2, they have shown a different response,—pollen being the most resilient in terms of viability. It was also observed that NO_2 was the gas with the most negative effect on pollen viability [17].

All of the above reveals two important considerations in relation to the impact that environmental stress has on the pollen grains of different plant species. On the one hand, not all the pollen types respond in a similar way to the presence of one or more environmental stress factors; on the other hand, the pollen grains of some species have a more sensitive response to these factors, so they could be used as bioindicators of adverse environmental conditions [16].

The objective of this work is to analyze the impact produced by different environmental stress factors, mainly air pollutants and UV-B, on the viability of the pollen grains of four species of ornamental trees frequently used in urban forests of Mediterranean cities. The results will reveal the reproductive health status of these species as well as which of them could be used as a specific bioindicator of a polluted environment.

2. Materials and Methods

Study Area. The study was carried out in Granada (37°11′17″ N; 3°36′24″ W), a standard medium-sized compact Mediterranean city, located in the southeast of the Iberian Peninsula, which extends over an area of 88 km^2 at the foot of Sierra Nevada, one of the highest mountain ranges in Europe (Figure 1). The climatic conditions are typical of the Mediterranean area, with a certain degree of continentality and high seasonal and intradaily temperature contrasts. The average annual temperature is 15.6 °C and the total average annual rainfall is 359 mm according to data provided by the Spanish Meteorological Agency, measured at the Granada airport weather station for the reference period 1981–2010 [18]. The study was carried out in 2015, which presented an average annual temperature of

16.6 °C and a total annual rainfall of 260.1 mm, clearly anomalous values in relation to those of the last reference climatic series.

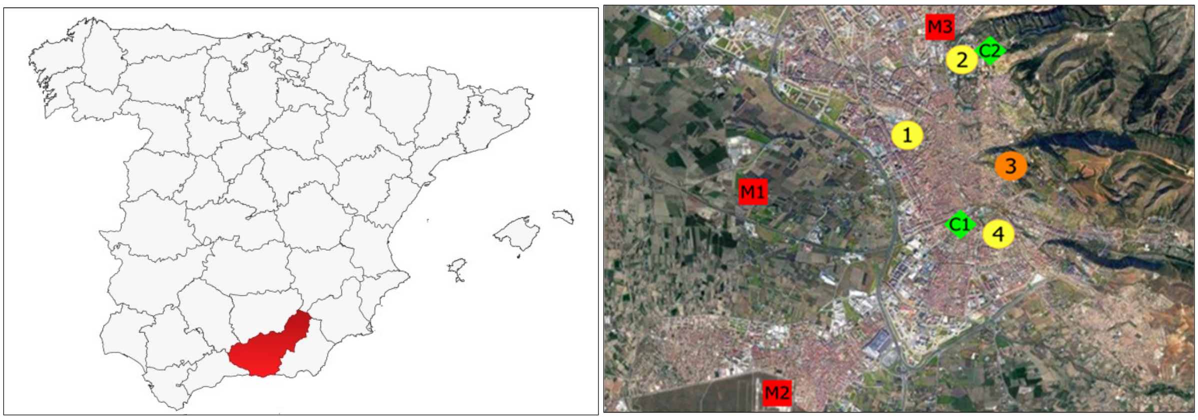

Figure 1. Location of the city of Granada and of the sampling points. 1: Fuentenueva Campus; 2: Cartuja Campus; 3: La Alhambra Forests; 4: Bola de Oro Sport Center; C1: Congress Palace station; C2: Cartuja Station; M1: IFAPA Station; M2: Air Base Station; M3: Cartuja Station.

In relation to the Urban Green Infrastructure, there are 363 green elements registered in the city, of which 341 of them have an extension smaller than 10.000 m^2 and an average of 4.74 m^2 of green area per resident [19]. The proportion of 160 trees per 1000 inhabitants is slightly higher than the European average [20]. The panel of most frequent plant species in the UGI elements exceeds 200 different species [21], a fact that is reflected in the diversity of pollen types found in the aerobiological analysis, which has been routinely carried out in the city since 1992, when the Granada station joined the Spanish Aerobiology Network [22]. Among the main genera contributing to the aeropalinological spectrum of the city are *Cupressus*, *Platanus*, *Ulmus*, *Populus*, with species widely represented in urban green areas. The concentrations of pollen from peri-urban emission sources are also relevant, forming both natural vegetation such as *Quercus* and *Pinus*, as well as large cultivated areas such as *Olea* [23].

The analysis of the city's historical pollen data series for the period 1992–2018 has shown that extreme pollen events, that is, those in which the daily concentrations of pollen grains are higher than the 99th percentile of the pollen records during this entire period, are increasing both in frequency and intensity in recent years [24]. It must be also added that the interactions established between pollen and atmospheric pollutants, in particular with NO_2, of which the levels recorded in Granada are among the highest in the entire Spanish territory, with average annual values that exceed not only 40 $\mu g/m^3$ but also the tolerance margin established at 20 $\mu g/m^3$ more [25,26].

Selection of plant speci0es and sampling areas. For this study, four of the most frequent tree species in the urban forests of the city of Granada and in numerous cities of the Mediterranean region [20,27] were selected: box elder (*Acer negundo*), European hornbeam (*Carpinus betulus*), Italian cypress (*Cupressus sempervirens*) and olive (*Olea europaea* [28]. The first three species present an anemophilous pollination strategy, for which the production and emission of pollen is very high [29], while in the case of the olive tree, although it presents an ambiphilous pollination strategy, that is, a mixed process between entomophily and anemophily [30], its extensive presence causes very high atmospheric pollen levels to be recorded, reaching more than 30% of the annual pollen content in the city's atmosphere [22,23].

For the purpose of this study, three urban areas were selected in which several of the following factors converged: (i) intense traffic areas, that is, those with an intensity of traffic of more than 1500 cars/hour according to the information regarding the traffic map of the city of Granada available at: http://www.movilidadgranada.com/tra_estado.php, access date: 15 November 2022; (ii) high exposure to direct solar radiation, from which ultraviolet-

B radiation (UV-B) values can be obtained; (iii) area exposed to adverse environmental conditions: high insolation, high/low temperatures, water deficit or wind gusts, based on data from the network of stations of the State Meteorological Agency (AEMET) in the city of Granada (https://x-y.es/aemet/prov-granada, access date: 15 November 2022); (iv) area of high urban density, based on data on the degree of urbanization defined by Eurostat, available at: https://ws089.juntadeandalucia.es/institutodeestadisticaycartografia/blog/2020/03/poblacion-y-espacio-degree-of-urbanization/, access date: 15 November 2022. In addition, an urban area that, due to its characteristics in terms of vegetation, low urban and traffic density, and absence of water stress, could be considered a control area was selected, that is, in which the impact of stress factors on pollen was minimal. These characteristics are brought together by the forests and gardens that surround the Alhambra Monument, the city's main urban forest in terms of extension and ecosystem services it provides [28,31]. Table 1 presents the characteristics of the different urban areas selected for this study and their location in the urban area.

Table 1. Sampling areas in the city of Granada. The reference station of the Surveillance and Control Network of Air Quality and of the State Meteorological Agency are indicated with numbers.

Sampling Area	Stress Factor	Reference Air Quality Station	Reference Meteorological Station
1. Fuentenueva Campus	i, ii, iii, iv	1	2
2. Cartuja Campus	i, ii, iii	2	1
3. La Alhambra Forests	CONTROL	2	1
4. Bola de Oro Sport Center	ii, iii	1	3

Reference air quality station: 1: Congress Palace station; 2: Cartuja Station. Reference meteorological stations: 1: Cartuja Station; 2: IFAPA Station; 3: Air Base Station. i: intense traffic area; ii: high exposure to direct solar radiation; iii: area exposed to adverse environmental conditions; iv: area of high urban density.

Data about the levels of three main pollutants: SO_2, NO_2 and CO were obtained in two stations of the Surveillance and Control Network of Air Quality, Regional Government of Andalusia (REDIAM), located in two areas of the city: one in the City Center (1: Palacio de Congresos) and another in the North area (2: Cartuja), for the period January–June 2015. The values of monthly average direct solar radiation in $10\times$ kJ/m^2 for the period January–May 2015, were provided by the State Meteorological Agency (AEMET). Ultraviolet-B (UV-B) radiation data were obtained adapting the model of Foyo-Moreno et al. [32] for the integrated daily values. This model uses only the global irradiation through the broadband hemispherical transmittance (kt) as input data, defined as the ratio between global irradiance and extraterrestrial global irradiance (both on a horizontal surface). This parameter is an indicator of the transparency of the sky, considering the most influencing factors in the determination of the solar radiation as aerosols and clouds. This model has wide applicability to other spectral regions as visible regions (400–700 nm) [33] and was also adapted to estimate erythemal ultraviolet irradiance [34].

Pollen harvesting. The flowers (cones in the case of *Cupressus* sp.) of the selected species were extracted in the period immediately prior to anthesis, that is, late January 2015 for *C. sempervirens*, February 2015 for *A. negundo* and *C. betulus* and late April 2015 for *O. europaea*. The optimal state of maturity of the pollen grains was verified by optical microscopy. A variable number of flowers were placed on an oven tray and dried at a moderate temperature (20 °C) to facilitate their extraction. Next, the flowers were placed on a sieve and gentle pressure was exerted to optimize pollen extraction and remove as many plant remains as possible from them. The amount of pollen obtained was approximately 2 g of clean pollen per species. Then, this was stored in plastic microtubes in a refrigerator at 4 °C, adequate temperature to keep them in dormant conditions in the event that the analyzes are carried out in less than a month [35,36]. The analyzes to determine pollen viability were carried out in February for the *Cupressus* samples, in March for the *A. negundo* and *C. betulus* samples and in May for the *Olea* samples.

Pollen Viability Determination. From the different techniques used to test pollen viability, staining tests are among the most advantageous, as they are faster and easier techniques to use [37–40]. Several studies also highlight that the determination of viability by staining techniques may correlate with the degree of in vitro-pollen germination, with the percentage of viable pollen generally being higher than that of germinated pollen [39,41,42].

In this study, staining with two different reagents was performed to determine the viability of pollen grains:

- Trypan blue. It measures cell viability by staining non-viable pollen grains a deep blue color [43]. This type of staining is based on the principle that living cells have an intact cell membrane, which is selective in relation to which compounds can cross it, that is, they exclude certain dyes, while dead cells do not [44].
- Pyrogallol red (Commercially sold as Redprot-aeromedi©). Evaluates the state of physiological maturity of the pollen grains, differentiating pollen grains with an intact and mature protein coat, which stain purple, from those that do not have the cover in an optimal state of maturity and therefore do not stain. If the pollen grain is mature, it will correspond to a more suitable phase for fertilization [45].

To determine the percentage of viability and maturity of the pollen grains, a small amount of pollen of each species was placed on a slide and 1 or 2 drops of Trypan blue or Pyrogallol red dye were added, and then covered with a coverslip. After 10′, enough time for the pollen to stain, the samples were analyzed by optical microscopy, scanning the sample from left to right and counting 500 grains of pollen in each of the prepared samples. For each type of pollen and zone, three independent repetitions in each of the three different slides were carried out, accounting for a total of 1500 grains of pollen. Trypan blue and Pyrogallol red staining allow easy differentiation of viable and mature pollen grains, respectively, when they appear intensely stained. However, when pollen grains stain weakly, it is necessary to establish selection criteria to distinguish between viable and non-viable and mature and immature. Thus, in the case of viability, viable pollen grains were considered those that did not present coloration and non-viable those that presented some degree of staining (Figure 2). On the other hand, for maturity, mature pollen grains were considered those that showed coloration and immature those that did not show coloration (Figure 2).

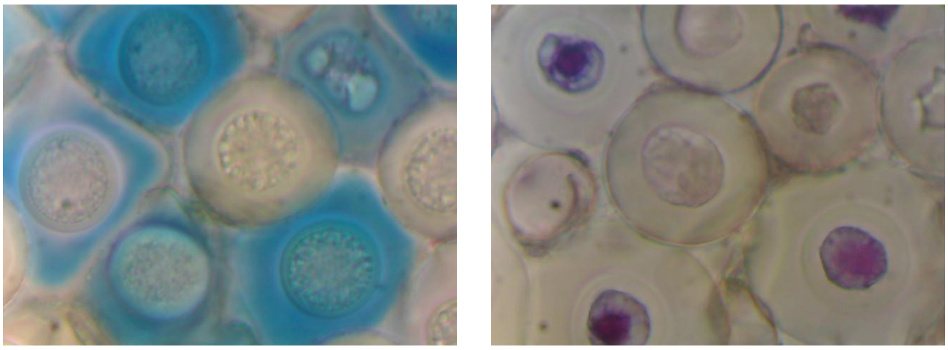

Figure 2. *Cupressus* pollen stained with Trypan blue (**left**) and Pyrogallol red (**right**).

Statistical Analysis. To determine differences between treatments and stress conditions, non-parametric Spearman's correlation analyzes were performed, since the data do not fit a normal distribution. Finally, to determine statistical differences between the control treatments and the samples, an analysis of variance (ANDEVA) and a Tukey HSD test (95%) were performed a posteriori. These methods aim to compare the individual means from an analysis of variance of several samples subjected to different treatments, this test allows discerning whether the results obtained are significantly different or not. To perform the statistical analyzes, the IBM SPSS Statistics software was used.

3. Results

In relation to air quality during the study period, the levels of three main pollutants SO_2, NO_2 and CO obtained in two stations of the Surveillance and Control Network of Air Quality (Table 2) showed that, for the period January–June 2015, NO_2 values exceeded the limit values for the protection of health. This limit established by Directive 2008/50/CE of the European Parliament and of the Council, of 21 May 2008, regarding the quality of ambient air and a cleaner atmosphere in Europe, transposed in Spain through the Royal Decree 102/2011, of 28 January 2011, regarding the improvement of air quality, limits the threshold values of an hourly limit value of 125 $\mu g/m^3$ for SO_2, an annual limit value for the protection of human health of 40 $\mu g/m^3$ for NO_2, and maximum eighth-hour average in one day of 10 mg/m^3 for CO.

Table 2. Monthly average values for pollutants SO_2, NO_2 and CO measured in two locations of the city of Granada during the study period.

	Granada Norte	**Palacio de Congresos Monthly Average Value**
	Monthly Average Value	**($\mu g/m^3$)**
$SO_2(\mu g/m^3)$	8.5	16.0
$NO_2(\mu g/m^3)$	47.3	36.3
$CO(mg/\ m^3)$	0.6	0.4

The values of monthly UV-B radiation in 10 kJ/m^2 for the period January–May 2015, are presented in Table 3. It can be seen how the average value of direct radiation increases as the year progresses.

Table 3. Monthly average UV-B radiation for the period January–May 2015.

UV-B (10 kJ/m^2)	
Month	**Average**
January	2.467
February	2.956
March	4.325
April	5.428
May	6.293

The results obtained to assess the viability of pollen from four tree species frequently used in the urban forest of Granada by specific stain are shown in Table 4. In the case of the samples stained with Trypan blue to determine viability, all the species in the control zone showed a higher percentage of viability than the pollen collected in the other sampled zones, that is, Fuentenueva Campus for *Acer negundo* and *Cupressus* spp., Bola de Oro for *Carpinus betulus*, and Cartuja Campus for *Olea europaea*. Viability percentages ranged from 56% (average of 303 $\pm$ 26 pollen grains in the three samples) for *Cupressus* to 85% (average 427 $\pm$ 11 pollen grains) for *Olea europaea* pollen. In the areas subject to different stress factors, it was observed that the percentage of viable pollen of *Acer negundo* and *Cupressus* was 20 (average 100 $\pm$ 61 pollen grains) and 39.5% (average 263 $\pm$ 30 pollen grains), respectively, while in the case of *Carpinus betulus* and *Olea*, the percentage of viability was close to 60% (average 294 $\pm$ 80 pollen grains for *Carpinus* and average 304 $\pm$ 80 pollen grains for *Olea*).

Table 4. Percentage of viability (Trypan blue) and maturity (Pyrogallol red) of the 4 pollen types considered in this study.

	Trypan Blue		**Pyrogallol Red**	
	Control	**Sample ***	**Control**	**Sample ***
	Viable (%)	**Viable (%)**	**Mature (%)**	**Mature (%)**
Acer negundo	60.5	20.0	82.8	88.9
Carpinus betulus	81.8	58.9	17.4	32.5
Cupressus spp.	56.0	39.5	31.7	49.3
Olea europaea	84.9	60.9	84.5	67.7

* Sample is Fuentenueva Campus for *Acer negundo* and *Cupressus* spp., Bola de Oro for *Carpinus betulus*, and Cartuja Campus for *Olea europea.*

The results that allow determining the maturity degree of the pollen grains by specific stain with Pyrogallol red revealed that *Acer negundo* and *Olea europaea* pollen in the control zone presented a percentage greater than 80% in both cases, 83 (average 414 $\pm$ 57 pollen grains) and 84% (average 445 $\pm$ 85 pollen grains), respectively. The optimal maturity degree was only presented by 17% (average 87 $\pm$ 58 pollen grains) of the *Carpinus betulus* pollen grains in this control area. However, the percentage of maturity for this species rose to 33% (average 163 $\pm$ 48 pollen grains) in the other urban locations, although it was also the lowest percentage of the four species sampled. The highest maturity percentage was that presented by *Acer negundo*, which was 89% (average 445 $\pm$ 84.5 pollen grains) of the sample collected in other areas the city.

Spearman's correlation analysis performed between the percentage of viable and non-viable pollen and the concentrations of atmospheric pollutants during the pre-flowering and flowering periods (Table 5) revealed that, in general, there is no effect of pollutants on the percentage of viable and non-viable pollen in the samples collected in the forests of La Alhambra, although in the case of *Carpinus* pollen, a significant correlation was found between non-viable pollen and UV-B radiation in February and SO_2 concentrations in March, and negative with CO concentrations in March. In the rest of the locations, the correlation of the viable pollen of *Acer* in the Fuentenueva location with the UV-B radiation of March, and the concentrations of CO in February, and those of the non-viable pollen of this same taxon, with a lower degree of significance, are noteworthy with the UV-B radiation of February and March. In the Fuentenueva location, a significant negative correlation is also obtained between viable *Cupressus* pollen and SO_2 concentration in February and non-viable pollen and UV-B radiation in March. The pollen sample from *Olea* from Cartuja showed a negative relationship with the UV-B radiation and the concentration of SO_2 and NO_2 in March.

In the case of the samples stained with Pyrogallol for the detection of maturity (Table 6), the effect of UV-B radiation in February and the concentration of CO in March for the pollen samples of *Acer negundo* from the Alhambra hardly stands out. *Cupressus* pollen sample collected in Fuentenueva was also significantly and negatively correlated with several pollutants in February and March.

Table 5. Spearman's correlation analysis between the percentage of viable and non-viable pollen (Trypan blue) and the concentrations of atmospheric pollutants during the pre-flowering and flowering periods.

Pollen Type	Location	Viability	UV-B February	UV-B March	SO_2 February	SO_2 March	NO_2 February	NO_2 March	CO February	CO March
Acer negundo	Alhambra	Viable	0.018	−0.102	−0.127	0.119	0.070	0.046	0.121	−0.013
		Non-viable	−0.126	0.257	−0.008	−0.126	0.204	0.086	0.141	0.118
	Fuentenueva	Viable	0.192	−0.064	−0.331	−0.078	−0.302	−0.331	**0.508** **	0.112
		Non-viable	**−0.412** *	**0.367** *	0.067	−0.053	0.052	−0.109	0.269	0.312
Carpinus betulus	Alhambra	Viable	0.089	−0.030	0.074	0.128	0.030	−0.071	0.074	−0.034
		Non-viable	**0.320** *	−0194	0.077	**0.543** **	−0.219	−0.041	0.028	**−0.483** **
	Bola de oro	Viable	−0.200	−0.311	0.003	**−0.423** *	−0.053	−0.083	−0.277	0.326
		Non-viable	0.101	−0.102	0.178	−0.262	0.106	0.078	−0.037	**0.444** *
Cupressus spp.	Alhambra	Viable	0.196	−0.199	−0.013	0.173	−0.245	−0.020	−0.099	−0.157
		Non-viable	0.1068	**−0.445** *	−0.078	0.199	0.027	0.199	−0.022	−0.151
	Fuentenueva	Viable	−0.105	−0.084	**−0.466** **	−0.075	−0.349	−0.100	−0.227	0.070
		Non-viable	−0.027	**0.388** *	−0.324	−0.177	−0.274	−0.186	−0.067	−0.003
Olea europaea	Alhambra	Viable	−0.409	−0.097	0.157	0.077	0.128	0.323	0.030	0.380 *
		Non-viable	−0.190	−0.086	−0.035	0.217	0.070	−0.050	0.142	−0.148
	Cartuja	Viable	0.295	0.240	−0.180	−0.099	−0.051	−0.080	−0.062	0.119
		Non-viable	0.025	**−0360** *	0.086	**−0.442** *	0.043	**−0.424** *	−0.103	−0.296

* The correlation is significant at 0.05. ** The correlation is significant at 0.01.

Table 6. Spearman's correlation analysis between the percentage of mature pollen (Pyrogallol red) and the concentrations of atmospheric pollutants during the pre-flowering and flowering periods.

Pollen Type	Location	Maturity	UV-B February	UV-B March	SO_2 February	SO_2 March	NO_2 February	NO_2 March	CO February	CO March
Acer negundo	Alhambra	Mature	**−0.173** **	−0.162	−0.214	−0.032	−0.084	−0.314	−0.013	**−0.356** *
		Immature	**0.054** *	−0.096	−0.241	−0.039	−0.190	0.424	−0.334	0.227
	Fuentenueva	Mature	−0.182	−0.372	−0.205	−0.081	−0.207	−0.289	0.101	−0.036
		Immature	0.100	−0.157	−0.229	−0.205	−0.120	−0.458	0.0	−0.422
Carpinus betulus	Alhambra	Mature	−0.02	0.0581	−0.270	0.161	−0.221	−0.228	−0.143	−0.176
		Immature	−0.016	−0.244	0.035	0.249	0.007	0.071	−0.093	−0.229
	Bola de oro	Mature	**−0.438** *	0.175	−0.160	0.027	−0.168	−0.007	−0.085	−0.265
		Immature	0.163	0.147	−0.086	0.051	0.017	0.038	−0.074	0.036
Cupressus spp.	Alhambra	Mature	−0.036	−0.076	0.182	−0.042	0.022	0.322	0.086	0.025
		Immature	−0.109	−0.110	0.330	−0.185	−0.094	0.119	−0.086	0.063
	Fuentenueva	Mature	**−0.238** *	0.179	−0.166	−0.327	−0.079	−0.279	−0.093	−0.103
		Immature	−0.042	−0.110	0.308	**−0.398** *	**0.387** *	**−0.391** *	0.348	**−0.501** **
Olea europaea	Alhambra	Mature	−0.064	0.177	0.086	−0.123	−0.051	−0.180	−0.193	0.068
		Immature	−0.129	−0.111	0.008	−0.123	−0.028	−0.204	0.088	0.132
	Cartuja	Mature	0.371	0.047	−0.006	−0.151	0.088	−0.104	−0.149	0.106
		Immature	0.377 *	−0.180	−0.088	0.332	−0.098	0.203	−0.299	0.148

* The correlation is significant at 0.05. ** The correlation is significant at 0.01.

4. Discussion

This study analyzed the effect of different environmental stress parameters on the viability of pollen grains from the tree species *Acer negundo*, *Carpinus betulus*, *Cupressus* spp. and *Olea europaea*, all of which are producers and emitters of large amounts of pollen, and which are frequently used as ornamentals in the metropolitan area of Mediterranean cities. It was based on the hypothesis that pollen viability decreases significantly when it is subjected to successive environmental stress conditions [46].

UV-B radiation (value obtained from the daily global irradiation data, [32–34] is one of the parameters analyzed that has the greatest effect on the viability of pollen grains, since UV-B has the potential to cause considerable damage to external protective structures, resulting in a decrease in the capability of germination [47]. The effects on growth rate and reproduction are also known [48,49]. In our study it has been observed that the pollen of species such as *Acer negundo* and *Cupressus* have been the ones that have shown a significant positive correlation between this variable and the percentage of non-viable pollen grains. In both species, the pollen is only protected from the outside by the wall of the pollen sac (cones in *Cupressus* and staminate catkins in *A. negundo*), so it is more unprotected than the pollen of plants with protective floral structures. A decrease in the germination rate of exposed *Acer negundo* pollen to NO_2 in contrast to non-exposed pollen has been demonstrated, even when the concentrations of pollutants to which it is exposed are below of the threshold of danger to human health [50]. In the case of *Cupressus*, an adjuvant factor to this effect could be the higher rupture rate that these pollen types present during their atmospheric transport in the environment case of *Cupressus* [51]. It should also be noted that both *Cupressus* and *Acer negundo* are winter flowering tree species [29], so the pollen maturation period coincided with those of maximum values of NO_2 in the atmosphere, whose values of January and February widely exceeded 40 micrograms/day due to the use of heating systems during the colder months, with monthly average values of 65 and 55 micrograms/m^3, respectively.

The *Olea europapea* pollen only showed a negative relationship with March UV-B radiation in the location Campus de Cartuja for non viable pollen. Although some studies have shown the protective role that some floral structures such as petals may have on pollen, March is too early for these structures to be fully developed. To this we must add that the Campus de Cartuja location is the highest of all the locations (780 m a.s.l. compared to 640 m a.s.l of the others), so the exposure to global radiation is also higher. In situations where the flowers are well fully developed, some studies have highlighted how floral structures can exclude up to 70% of radiation in environments where exposure is very intense, such as high-altitude areas [52]. This percentage can even increase up to 98% in the case of those types of pollen that contain flavonoids in their wall. These compounds, in addition to being responsible for the yellow color of pollen, which plays an important role in attracting insects, have been shown to have a relevant protective effect against oxidative stress, and even increase synthesis as a defense measure against changes in UV-radiation [53]. In the case of olive pollen, its flavonoid content can be up to 422 ppm, much higher than the average content of this compound in Angiosperm pollen grains, which would confirm the greater protection of pollen against the effects of UV-B radiation [54].

Motorized traffic and biomass burning are the main sources of emission of atmospheric pollutants such as carbon monoxide (CO) and sulfur dioxide (SO_2) in the study area [23,25]. Although all species had their viability affected by almost 50%, again both *Acer negundo* and *Cupressus* sp. were the most affected pollen types, with losses of up to 60% of viable pollen grains. Some studies have already highlighted the qualitative and quantitative changes that can occur in the characteristics of the pollen grains of these species when they are subjected to different concentrations of polluting gases [55]. In the case of some Cupressaceae, differences have also been found in the chemical composition of the pollen grains collected in areas with different levels of air pollution [14].

One factor that may play a role in this is the primitive nature of the *Cupressaceae* pollen grains, and their morphology, which is very fragile to changes in environmental humidity, to which it reacts in the form of rupture, and therefore, with loss of viability [56]. This situation is favored by the high number of existing specimens in the city, due to its consideration as an emblematic tree [31], as well as the very high pollen production per specimen, calculated at more than 275×10^9 [57]. Then, in addition to the pollen fragmentation due to changes in environmental humidity, mechanical fragmentation caused by atmospheric dynamics can occur, especially during the increasingly frequent episodes of extreme pollen events in which daily concentrations exceed 1500 pollen grains/m^3 [24].

However, in the case of the olive pollen, no clear relationship has been found between the levels of atmospheric pollution and the structural change of the organic compounds present in the olive tree pollen wall [58]. This could be related to the improvement to which this species has been subjected in order to increase its agricultural yield, which has reinforced its resistance to different types of environmental stress, among them reinforcing the synthesis of protective flavonoids as already mentioned above [59,60]. It should be noted that certain studies confirm that some of the proteins that make up the pollen wall, are not expressed in some of the agricultural varieties subject to genetic improvement [61]. These differences are maintained over the years, and are intrinsic to the genetics of each cultivar [62]. Although with our results we cannot confirm the molecular changes experienced in the protein coat, we have been able to observe how olive pollen grains have been the least affected both in maturity and in viability, confirmed by their high rate of fruiting in the area [63].

In relation to maturity, only in the *Cupressus* pollen there is evidence that even at ambient NO_2 concentrations far below the value at risk for direct effect on the respiratory health, some of the proteins in the pollen wall may stop being expressed, even when these pollen grains are still in the air inside the pollen sacs [64]. Therefore, the pollen emitted after anthesis already has defects in terms of its maturity phase affected by NO_2. According to studies by Cuinica et al. [8], the viability, germination and total soluble proteins of pollen exposed to NO_2 decrease significantly when compared to species not exposed to this pollutant. This could be due to the fact that, although NO_2 levels were sometimes above the critical value for the protection of vegetation, established at 40 $\mu g/m^3$, the exposure time may not be sufficient to cause damage that would affect pollen maturity, leaving the pollen grains protected by the anthers [55]. Some studies also indicate that the reaction of pollen from different species to the same concentration of NO_2 is not the same, presenting different germination rates [8,65]. The maturity results obtained for *Carpinus betulus* are also striking, since it is the species that has shown a lower percentage of maturity both in the control zone and in the rest of the zones, but also being the percentage of maturity of the zone of lower control than that of the other zones. Although some studies have revealed that viability, germination and total soluble protein decrease significantly when pollen of this species is exposed to low levels of various pollutants (NO_2, CO, O_3, SO_2) [30,64,65], in our results, no significant correlation was obtained with any contaminant. There is evidence of a physiological and biochemical response that these trees may have in the face of a high presence of atmospheric NO_2, including changes in soluble protein [66], but in our case we believe that the low maturity and quality of its pollen may be more related to weather conditions. The hornbeam is a mesophilic species of temperate climates in which the high summer temperatures limit its distribution. In Europe it is widely distributed naturally in the central part, but its presence is practically null or marginal in the Iberian Peninsula [67]. However, and although there are several species and varieties that are cultivated in Spain [68], there is also evidence of their vulnerability to heat stress [69], a situation that has worsened in the study area with episodes of waves of more frequent and intense heat in the last 15 years [70].

5. Conclusions

Our results have highlighted that the different environmental and pollution conditions of the areas of the city in which the trees are located will have an influence on the levels of stress that affect them, and consequently, on the viability of the pollen grains of the different tree species. Of the different areas considered in this study, the trees that form the forests of the Alhambra have been the least affected by the parameters and variables evaluated, presenting the highest viability rate, greater than 56%. On the contrary, the pollen of the tree species in the Cartuja and Fuentenueva areas, both with important sources of polluting emissions due to intense traffic, present a viability of less than 40%, with the UV-B and SO_2 parameters having the greatest effect on both viability and maturity. Pollen from *Olea europaea* has had the least impact due to the different stress parameters, while *Carpinus betulus* and *Cupressus* have been the two most affected species. In both, clear differences were observed between the samples from the different areas of the city and the one from the forests of La Alhambra, both presenting percentages of non-viability and non-maturity above 50%. These species, in addition to *Acer negundo*, are winter flowering, so the coincidence of the pollen maturation period in the reproductive structures with the maximum emission of pollutants derived from heating and traffic systems into the atmosphere may also be a factor influencing the viability of pollen grains.

We can conclude that the effect of environmental parameters and pollutants on the viability of pollen grains differs according to the species/genus and according to the conditions of the areas considered. In addition to the importance that the loss of quality of the pollen grain can have in the reproductive processes of plants, the monitoring of the response that the viability and germinability of the pollen of certain species (i.e., *Cupressus* spp. and *Carpinus betulus* in our study area) is having before certain stressful environmental conditions, can be used as a bioindicator and prevent the impacts that in the case of not taking action can affect other species.

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