

Phenological analysis of grasses (Poaceae) as a support for the dissection of their pollen season in Perugia (Central Italy)

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Received: 16 September 2016 / Accepted: 11 January 2017
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Abstract Grasses (Poaceae) pollen is a major cause for allergic diseases worldwide. Pollen monitoring in the atmosphere is of primary importance for symptoms interpretation and therapy planning. Microscopic pollen identification and counts do not allow the detection at species or genus level because of the stenopalynous nature of the family. Nevertheless, the assessment of the flowering phenology of different species would be important, because not all grass allergens are cross-reacting and allergic patients could be differentially sensitized. In this work, a phenological survey was carried out in five stations located on the urban territory of Perugia (Central Italy), from April to September 2015, recording the alternation between flowering phenophases of 19 grass species and estimating their contribution to the airborne pollen load of the area through the calculation of a Phenological Index. Moreover, pollen grains of the different species were collected and observed, confirming the impossibility to make a discrimination during microscope pollen counts. The prevailing grasses in terms of

contribution to the pollen detection in the studied area resulted to be *Dactylis glomerata* and *Lolium perenne* during spring and early summer, and *Cynodon dactylon* during late summer. Data should be validated repeating the survey in successive years and possibly using biomolecular tools, but the obtained information could be relevant for diagnosis and treatment of grass pollen allergies.

Keywords Poaceae · Allergy · Phenology · Pollen · Flowering · Central Italy

1 Introduction

The Poaceae family counts more than 600 genera, comprising over 10,000 species of which around 450 can be found in Italy (Pignatti 1982; Watson and Dallwitz 1992). Their distribution varies largely from one area to the other, because of the influence of microclimatic environment, geography and also human management actions. They include annual and perennial species, which mostly exploit wind as vector for pollen transport. As the majority of anemophilous plants, their inflorescences are characterized by a high number of flowers producing multitudes of small and dry pollen grains which are released in the atmosphere and can be transported for kilometers.

Their ubiquity and large amount of pollen produced make these species the main cause of pollinosis across

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Europe (D'Amato et al. 2007). For this reason, aerobiological monitoring represents an essential support for medicine, when it is used for allergy diagnosis and therapy planning. Classic aerobiological monitoring is usually carried out with Hirst-type air samplers (Hirst 1952), which permit the airborne pollen identification and count by optical microscope analysis as grass pollen. Unfortunately, this method does not allow the discrimination of pollen grains belonging to different wild grass species (pollen from cultivated species is bigger and not aerodynamic, thus not much present in atmosphere), because they are stenopalynous *taxa* characterized by only a slight variation in pollen morphology (Erdtman 1952; Perveen 2006). Joly et al. (2007) proposed a biostatistical approach to discriminate between pollens from cereals and wild grasses. It was based on the execution of a large number of measurements on pollen and *annulus* diameters, in order to establish size thresholds related to grains belonging to different grasses. Anyhow, this system is still far from a species or genus-specific classification of grass pollen grains based on morphology. In fact, pollen curves and calendars obtained by routine aerobiological monitoring usually refer generically to “grasses” and do not describe the succession of flowerings of the several species during the long pollen season (early spring to late summer).

Phenological analysis, consisting of field observation and timing of recurrent biological phases of plants, has already been used to integrate airborne pollen in various genera, especially woody ones, but also herbaceous *taxa* (Latorre and Bianchi 1998; Jato et al. 2002; Hidalgo et al. 2003; Kasprzyk 2003; Guardia and Belmonte 2004; Orlandi et al. 2005; Estrella et al. 2006; León-Ruiz et al. 2011; Tormo-Molina et al. 2015; Cebrino et al. 2016; Kmenta et al. 2016a, b). Thus, it could well support aerobiological data in the dissection of Poaceae pollen season in the area of Perugia (Central Italy). The main application of this information would be in the allergen-specific immunotherapy (SIT). While knowing the causative species is not so important when allergy is treated with symptomatic drugs, it becomes relevant when vaccines are prescribed (Frenguelli et al. 2010). It is true that there is a high cross-reactivity between the allergens of many grass species (Ferreira et al. 2004; Johansen et al. 2009) but exceptions exist, especially between allergen subsets characterizing species

belonging to different sub-families (e.g., between Pooideae and Chloridoideae) (Andersson and Lidholm 2003; Davies et al. 2005). Nowadays, a mix of extracts from the most widespread and allergenic species is used for SIT, and it seems to be the better strategy to reflect the natural exposition of subjects that are mostly polysensitized (Peltre 2007). Integrating aerobiological and phenological approaches could help in correlating symptoms with the particular timing of species-specific pollen seasons, implementing immunospecific therapy and component-resolved diagnosis (Valenta et al. 1999) with the aim of making clinical evaluations and following treatments more effective and personalized. In this sense, phenological observations are essential to establish the behavior of the different Poaceae species to determine which of them mostly contribute to the pollen curve in a given area (León-Ruiz et al. 2011). A few authors have already drawn up a list of major local Poaceae species displaying a positive correlation between phenological development and pollen concentrations (Zerboni et al. 1991; Newham et al. 1995; León-Ruiz et al. 2011). Zanotti and Puppi (2000) carried out a detailed phenological survey of spontaneous grasses populating the neighborhood of Bologna (Italy). Frenguelli et al. (2010) assessed flowering times of twelve grass species through a phenological analysis conducted on the whole national territory, evidencing differences in the behavior of the *taxa* in the North, Centre and South of Italy.

This study has been focused on the urban territory of Perugia and aims at the dissection of Poaceae pollen season. In particular, phenological analysis was used to individuate the most relevant grass species in terms of contribution to the total pollen load of the atmosphere and subsequent allergenic potential.

2 Materials and methods

Daily grass pollen concentrations were obtained through the routine aerobiological monitoring activity: Pollen sampling was carried out with a Hirst-type trap placed on the roof of the Department of Agricultural, Food and Environmental Sciences (Lat. 43°6'5''N—Lon. 12°23'45''E, 465 m above sea level) in Perugia (central Italy). The pollen season start and end were calculated using the 5% criterion (Nilsson

and Persson 1981). Pollen grain has been expressed as average of daily pollen per cubic meter of air (p/m^3).

For the phenological observations, five sampling stations were selected in an area of about 25 km² on the urban territory. The distances of the stations from the sampler were comprised between 180 m and 4.70 km; their altitude ranged from 185 to 465 m above sea level (Fig. 1). The stations represented different urban green spaces (mostly parks, public gardens and border zones) characterized by a similar plant coverage: They were populated, among other *taxa*, with several species of spontaneous grasses. The specific cover surface of this kind of vegetation can change 1 year to another due to natural factors or, for example, to the recovery of marginal and uncultivated areas carried out by man. For these reasons, it is not possible to estimate the surface occupied by the single species, but only to identify those which are most

frequently found in different spots of the city (prevailing vs. non-prevailing, see further). Due to all these factors, it was proposed to simplify the model assuming the equal contribution of each station to the monitored airborne pollen cloud.

The observations were conducted referring to the Marcello's phenological stages (Marcello 1953) and then translated into Biologische Bundesanstalt, Bundesortenamt und Chemische Industrie (BBCH—Table 1), over a period of 5 months and a half (April–September 2015), during which sampling was repeated each 10–15 days. For each species, 25 specimens were checked, and the phenophase of the majority of them was assigned. The observed species are listed in Table 2. For most of them, some inflorescences were collected during pollen emission; microscope slides were prepared staining pollen with gelatin-fuchsin solution and then observed and

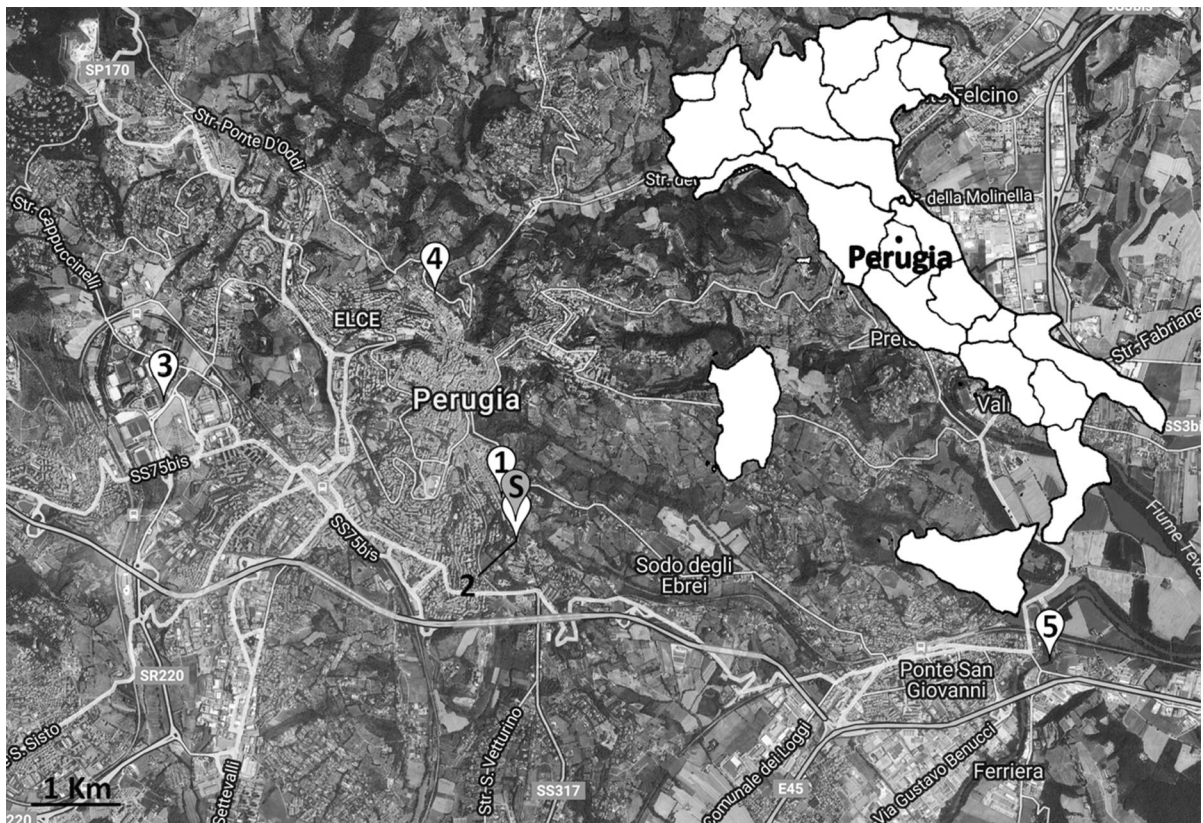


Fig. 1 Area covered by the survey. *S* Hirst-type pollen trap. Roof of the Department of Agricultural, Food and Environmental Sciences, 465 m a.s.l.; *1* Department garden, 450 m a.s.l.; *2* Porta San Costanzo, street border, 440 m a.s.l.; *3* Pian di

Massiano, untamed fields near Palaevangelisti (sports arena), 275 m a.s.l.; *4* Parco Sant'Angelo, public park, 465 m a.s.l.; *5* Ponte San Giovanni, bank of river Tevere, 185 m a.s.l. (<https://www.google.it/maps>)

Table 1 Phenological key used to identify grass phenophases, translation in BBCH scale stages and scores assigned to each phase for the calculation of Phenological Index

	Marcello's key symbol	Description of the phenophase	BBCH corresponding stages	Score
1	000	Absence of visible inflorescence	<55	0
2	+00	Inflorescences partially or fully emerged: stamens not extruded	55–59	0
3	++0	Stamens partially extruded	60–62	1
4	+++	Full flowering: all stamens extruded, full pollen emission	63–65	2
5	0++	Part of stamens withered	66–68	1
6	00+	All stamens withered	69–70	0
7	000	Beginning of the fructification	70<	0

Table 2 *Taxa* of wild grasses identified in the phenological survey

	<i>Taxon</i>	Common name	Life cycle	Presence
	<i>Agropyron</i> spp. Gaertn.	Wheatgrass	P	<
	<i>Alopecurus mysuroides</i> Huds.	Black grass	A	<
	<i>Avena fatua</i> L.	Common wild oat	A	>
	<i>Brachypodium</i> spp. (L.) P. Beauv.	False brome	A	>
	<i>Bromus</i> spp. L.	Brome	A	>
	<i>Cynodon dactylon</i> (L.) Pers.	Bermuda grass	P	>
	<i>Dactylis glomerata</i> L.	Orchard grass	P	>
	<i>Digitaria</i> spp. Haller	Crabgrass	A	>
	<i>Echinochloa crus-galli</i> (L.) P. Beauv.	Common barnyard grass	A	<
	<i>Eragrostis cilianensis</i> (All.) Vign. ex Janchen	Gray lovegrass	A	<
	<i>Festuca</i> spp. L.	Fescue	P	<
	<i>Holcus lanatus</i> L.	Yorkshire fog	P	>
	<i>Hordeum murinum</i> L.	Wall barley	A	>
	<i>Lolium perenne</i> L.	Perennial ryegrass	P	>
	<i>Phalaris</i> spp. L.	Canarygrass	P	<
	<i>Poa annua</i> L.	Annual meadow grass	A	>
	<i>Poa pratensis</i> L.	Kentucky bluegrass	P	<
	<i>Poa trivialis</i> L.	Rough bluegrass	P	>
	<i>Setaria</i> spp. P. Beauv.	Foxtail	A	<

P perennial, A annual; > *taxon* found in 3 or more stations (prevailing), < *taxon* found in less than 3 stations (non-prevailing)

photographed in order to make a rough morphological comparison among grains of different species and confirm the impossibility to distinguish them.

A Phenological Index was elaborated to quantify the contribution of the surveyed species to the atmospheric pollen load of the city and correlate the field observations with aerobiological data. The index was calculated as follows: Depending on the phenological stage, a score proportional to pollen emission in such phase was assigned to every *taxon* at each observation in the various stations (Table 1). Perennial species have been recognized to produce a larger

amount of pollen than annual ones (Prieto-Baena et al. 2003). Since the sampled grasses were a mix of the two types, and not all of them had the same distribution among the stations (reflecting a probable different presence in the whole urban area) (Table 2), the scores were adjusted as shown in Table 3. The daily Phenological Indexes were calculated summing final scores assigned to each *taxon* in each station. Single-species (or -genus in some case) Phenological Indexes were also obtained to estimate the contribution of each of them to the grass airborne pollen in the city of Perugia. Since this is a pilot study, and no similar index has

Table 3 Score adjustment based on the life cycle and the different distribution of the surveyed grasses

Grass characters	Annual	Perennial
Non-prevailing	Simple Score	Score X2
Prevailing	Score X2	Score X4

been developed previously in bibliography, a method was needed to validate the goodness of Phenological Index as an estimator of the grass pollen presence in the atmosphere. The chosen way was to compare Phenological Index values to airborne grass pollen concentration for the same days, by means of correlation analysis.

3 Results

3.1 Aerobiological analysis

In 2015, the grass main pollen season in the area of Perugia started on 25 April and ended on 27 July, lasting in total 94 days. Nonetheless, phenological sampling was prolonged until September (when few grass pollen grains were still captured by the sampler) to investigate the relative contribution of late flowering species to the right tail of the grass pollination curve. The peak in concentration was reached on 29 May.

3.2 Phenological analysis

The selected dates of sampling for comparing daily grass pollen concentration and Phenological Index during the same days are reported in Table 4. The two series have a high correlation coefficient ($r = 0.934$, $p < 0.001$), and this is also shown also in Fig. 2. Figure 3 illustrates how the pattern of pollination monitored by the aerobiological sampler is strictly linked with the Phenological Index used to describe the field situation of our species of interest. Using single-species/genus Phenological Indexes, a “decomposition” of the phenological curve could also be extrapolated. Figure 4 shows the contribution of each *taxon* to the total airborne grass pollen during the season, estimated through Phenological Indexes. *Taxa* with higher specific Phenological Index were identified as principal sources of the total grass pollen in the atmosphere of Perugia: *Dactylis glomerata*, *Lolium*

perenne, *Poa trivialis* and *Cynodon dactylon*. Table 5 reports the percentages represented by their indexes on the total. Phenological analysis also allowed assessing how their contribution in terms of pollen released is distributed during the season: Among the four species, *Dactylis* is the first to flower, followed by *Poa* and *Lolium*, whose pollen seasons are almost simultaneous. The superimposition of flowerings ends with *Cynodon*, which is the main source of grass pollen in late summer (Fig. 5).

3.3 Morphological observations on pollen

Pollen of 17 *taxa* out of 19 analyzed (*Hordeum murinum* and *Poa annua* pollen quality was not good enough to photograph) were observed at optical microscope, just for a rough morphological comparison. Pictures are shown in Fig. 6. The possibility to discriminate the species could be verified with a biostatistical analysis on the average diameter of the pollen grains of each *taxon*, but the mere microscope observation suggests that such measurements would not be very reliable, given the high size *intra*-group variability.

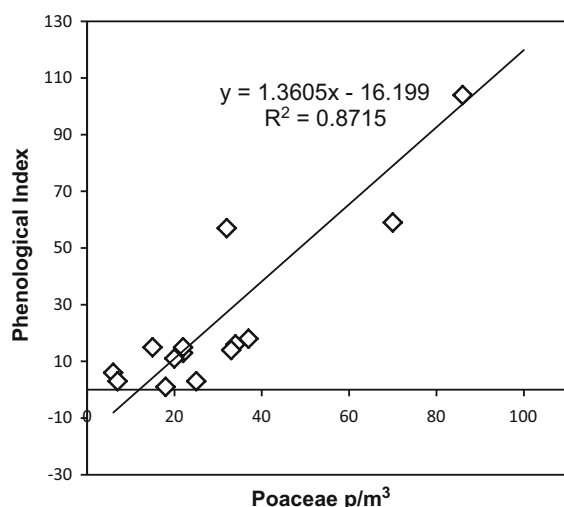
4 Discussion

Except for a slight anticipation in the beginning (which seems to follow a trend recently observed), the seasonal course of Poaceae flowering in Perugia, 2015, was quite in line with the average (Ghitarrini et al. 2016).

All the representative genus/species in the urban area (not only allergenic ones) were introduced in the analysis to have a complete overview on the different possible contributors to the total grass airborne pollen of the city of Perugia. The high correlation coefficient ($r = 0.934$, $p < 0.001$) with pollen concentration series and patterns shown in Fig. 3 demonstrates that our Phenological Index could be a good estimator of the different contribution of various grass species on the airborne grass pollen load in our area of interest, and gives an idea of the order of their flowerings succession. According to Fig. 3, there are just three periods of the season in which the patterns seem to diverge: First, a strong reduction of the airborne pollen concentration is evident at the end of May, right before the peak, while Phenological Index stays high. The

Table 4 Pollen counts and Phenological Index series for the dates of sampling

Date of survey	Poaceae pollen grains/m ³	Phenological Index
2 April	6	6
13 April	13	22
24 April	16	34
5 May	104	86
15 May	139	100
25 May	59	70
5 June	57	32
16 June	15	15
26 June	14	33
6 July	18	37
17 July	15	22
28 July	11	20
11 August	1	18
27 August	3	25
14 September	3	7

**Fig. 2** Correlation between Phenological Index and grass pollen concentrations for the same days

explanation is quite intuitive, since a similar drop of the pollen concentrations at that time of the year is often ascribable to weather events. In fact, checking meteorological databases (ilmeteo.it) confirmed the occurrence of heavy rainfall on four consecutive days between 22 and 26 May.

Also, Phenological Index does not show the peak that is notable for pollen concentrations at the beginning of June. The causes for this incongruity could be various, for example the peak could have been determined by a wind directed re-lifting of pollen

previously emitted, the grains could come from a distant source, and maybe an additional phenological sampling between 5 and 16 June could have given a better fitting comparison. Another discrepancy is notable at the end of the season, where Phenological Index seems to overestimate the presence of pollen in the atmosphere. This is probably due to the considered contribution of *Cynodon dactylon*, which is the predominant species at this time of the year in all the stations. Even if it is perennial, Prieto-Baena et al. (2003) and Aboulaich et al. (2009) found out that its production of pollen grains per inflorescence is quite low and more similar to annual grasses. So maybe the quadrupled score assigned to this species is not representative of the real amount of pollen it produces and releases. Another possibility is linked to an increased incidence of rainfall in the middle of August (ilmeteo.it), which could have lowered the atmospheric pollen load.

The single-species (-genus) Phenological Indexes for the whole period covered by the survey reveal that four species alone contribute to more than 50% of the airborne grass pollen (Table 5). Within them, *Dactylis glomerata* and *Lolium perenne* are surely the ones which deserve more attention, given their high ubiquity and allergenic potential (pollenlibrary.com). Furthermore, these species have been found to be between the major contributors not only in other studies regarding the Mediterranean area (León-Ruiz et al. 2011; Cebrino et al. 2016), but also in Central–

Fig. 3 Comparison between trends of Phenological Index and daily pollen concentrations (p/m3) during the period of the study (mm3 = 3-day simple moving average)

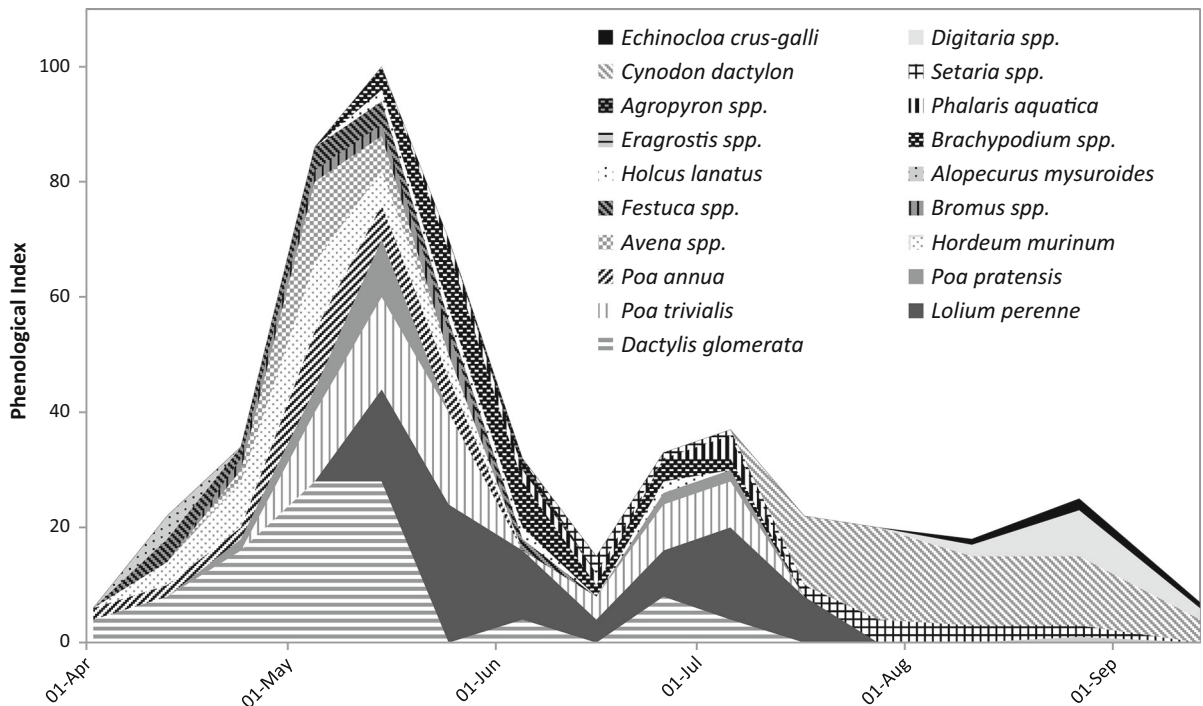
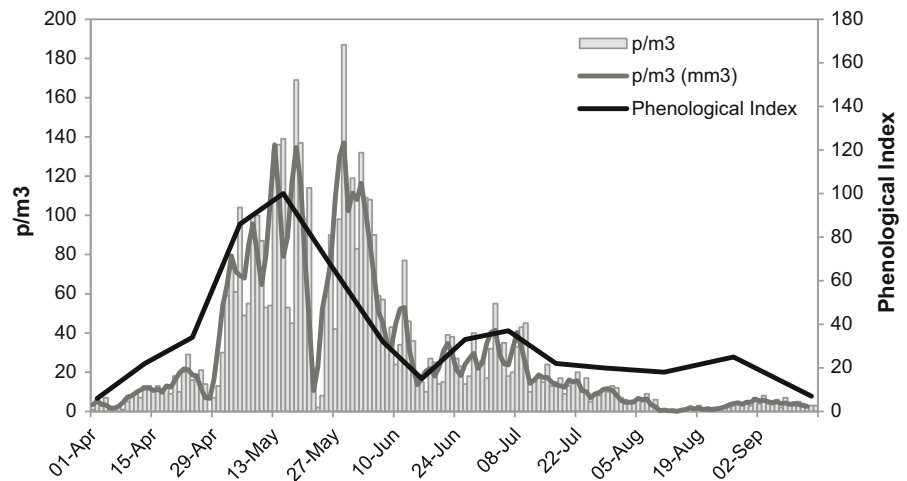


Fig. 4 “Decomposition” of the Phenological Index trend, evidencing the contribution of single *taxa*

Northern Europe (Kmenta et al. 2016a, b). *Poa trivialis* also has been found in almost all the sampling stations, but its pollen is classified as moderately allergenic (pollenlibrary.com), and it is not usually deemed to be problematic for allergic patients. As already said, the *Cynodon dactylon* percentage on the total Phenological Index could be an overestimation. Anyway it is known as a severe allergy-causing factor, is extremely diffused in the urban area of Perugia and thus should be

taken into consideration as the most probable elicitor of late summer allergic manifestations.

The microscope observation of the pollen grains collected during the survey confirmed the stenopalynous nature of the Poaceae family, characteristic that does not allow a species or genus-specific identification and count of the pollen grains detected in the samples. The phenological survey instead helps to establish the succession of flowerings of the various

Table 5 Grasses that most contribute to the airborne pollen load in Perugia

Species	Contribution on Total Phenological Index (%)
<i>Dactylis glomerata</i>	18.98
<i>Lolium perenne</i>	16.70
<i>Poa trivialis</i>	12.14
<i>Cynodon dactylon</i>	10.63

species, which carry different allergen subsets and could be differently sensed by allergic subjects.

It has to be taken into account that the study has been conducted on a single year of observations, and it should be repeated to eliminate casual effects and define the trend. Moreover, since the sampling stations were selected and considered equivalent in terms of contribution to the pollen cloud monitored by the trap, enlarging the area of the survey, as well as the number of surveyed sites, would probably add accuracy to the analysis.

In fact, as earlier stated, the possibility of long distance transport of pollen should not be ignored. For example, no populations of Timothy grass (*Phleum pratense*) were detected in the sampling stations, and it can be supposed that it is not present in the whole urban area, because at this latitude it usually prefers higher altitude. Anyway the presence in the atmosphere of *Phleum* pollen and/or allergens (known as capable of eliciting allergic reactions in most of the grass-sensitized patients—Scaparrotta et al. 2013) cannot be excluded, as they could be transported by air masses moving from adjacent areas where it grows spontaneously or is cultivated as forage. A possible way to integrate data could be the biomolecular analysis of airborne grass pollen DNA, and generally, molecular tools could be exploited to validate the superposition of Poaceae pollination and phenological curves proposed in this study (Ghitarrini et al. 2015).

5 Conclusions

The urban area of Perugia (Central Italy) is populated with a wide range of spontaneous grasses, both allergenic and non-allergenic ones, which flower mainly between April and September. Since Poaceae is a stenopalynous family, the information given by

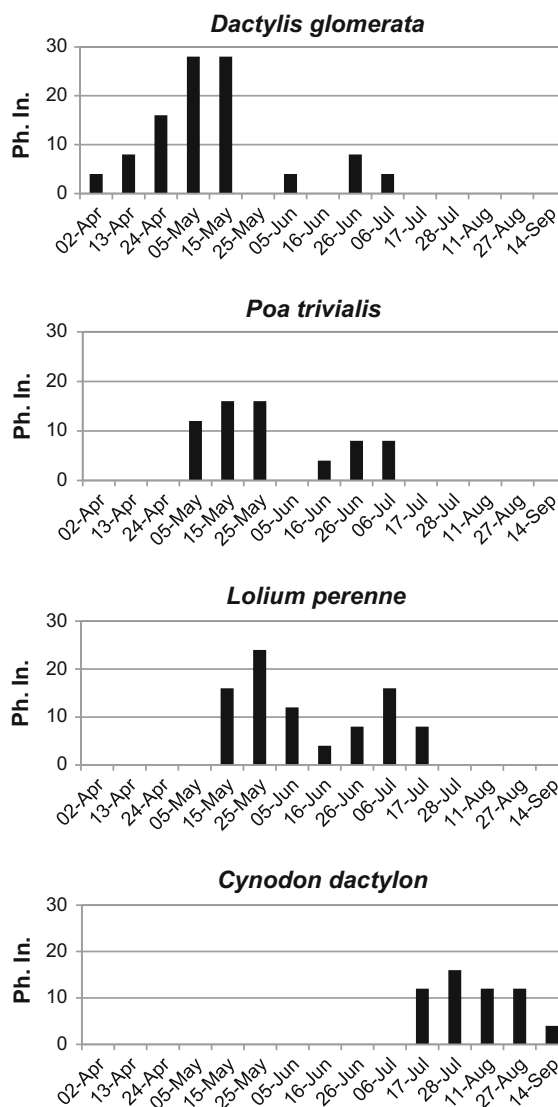
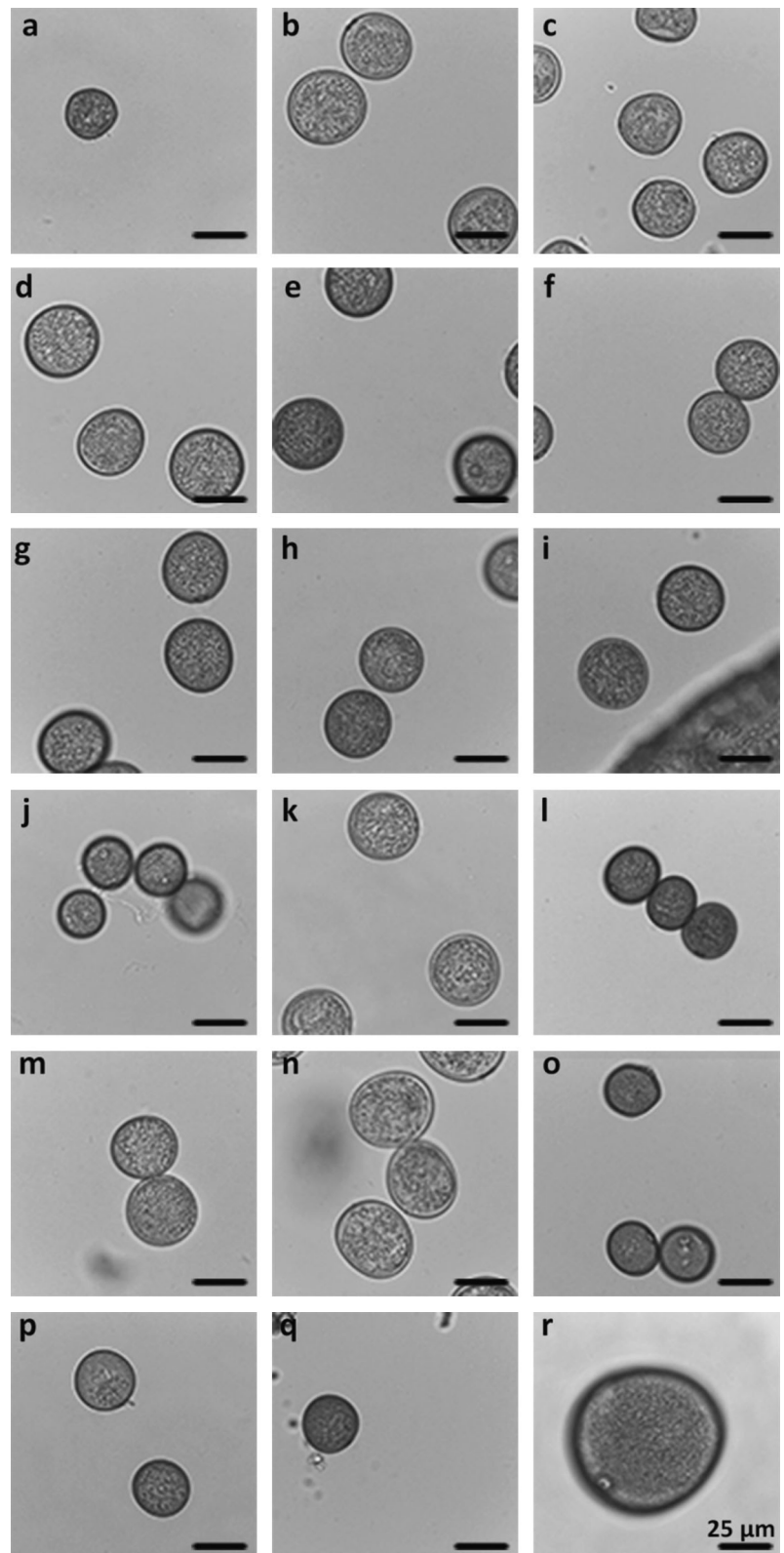


Fig. 5 Pattern of Phenological Indexes of the four species that mostly contribute to the grass pollen load of the atmosphere in Perugia. As shown, their flowerings overlap covering the whole period of the survey

classical aerobiological monitoring (daily cumulative concentration of grass pollen per cubic meter of air) should be integrated with field phenological observations of the different species present in the area of the study (which are not simultaneous), in order to obtain a preliminary indication of how and when the pollination curves of single species/genera overlap. This may be relevant in the interpretation of allergic symptoms during the season and may explain why in some grass-sensitized patients the occurrence and

Fig. 6 Poaceae pollen grains photographed at optical microscope with a magnification of 630X.

a *Agropyron* spp.;
b *Alopecurus mysuroides*;
c *Avena fatua*;
d *Brachypodium* spp.;
e *Bromus* spp.; **f** *Cynodon dactylon*; **g** *Dactylis glomerata*; **h** *Digitaria* spp.;
i *Echinocloa crus-galli*;
j *Eragrostis cilianensis*;
k *Festuca* spp.; **l** *Holcus lanatus*; **m** *Lolium perenne*;
n *Phalaris* spp.; **o** *Poa pratensis*; **p** *Poa trivialis*;
q *Setaria* spp.; **r** *Zea mays* (being a domesticated species, it has bigger and quite recognizable pollen grains that are rarely transported far from the emitting plant and usually do not elicit allergic responses). *Hordeum murinum* and *Poa annua* pollen grains could not be photographed



severity of respiratory symptoms not always coincides with the standard pollen concentrations. In addition, this kind of analysis could be of central importance for a correct choice and timing of vaccines in allergen-specific immunotherapy, as well as in component-resolved diagnosis. The collected data in fact suggest that not all the detected grasses have the same importance in the sensitization process, identifying those to which more attention should be paid. In particular, it was found that in Perugia the most allergologically harmful species are *Dactylis glomerata* and *Lolium perenne* between April and mid-July, while *Cynodon dactylon* could be linked to late summer allergic symptoms. The consistence of this approach should be further investigated, repeating the survey in different years and areas, and a biomolecular approach could be used to validate results.

Moreover, this phenological information could help in dissecting the effects of climate change on the grass pollen season. It has been demonstrated that in the last decades, cumulative grass pollen season has undergone some modification probably linked to the rising temperatures. Since Poaceae pollen curve is a sum of those belonging to several species flowering in the area, a phenological data integration could help to better understand whether and how the species are responding differently to climate change.

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