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Impact of climate change and management on allergenic pollen of wind-pollinated species

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Abstract

Pollen allergy is a widespread disease, and in the context of climate change an increase in prevalence as well as in allergic symptoms can be expected. Among the wind-pollinated plant species, the tree species birch and alder, the shrub species hazel, and the grass family play a central role in allergies.

In this PhD thesis, allergenic pollen was studied in various field experiments and by analysis of existing data in the context of agriculture management and climate change. The focus was mainly on grasses, specifically of the *Poaceae* family, due to their high allergenic potential and ubiquitous distribution. The results were published in four publications.

The grass family is characterized by a large number of species, which are divided in agriculture into numerous cultivars, whose differences in allergen content are still relatively little studied. Therefore, seven grass species and 15 cultivars were investigated and compared in the first study by Jung et al. (2018). The aim was to determine the extent to which the species and their cultivars commonly grown in agriculture differ in their pollen characteristics. The laboratory analysis focused primarily on the determination of the allergen content. Protein content, pollen weight and pollen production were also quantified. Allergen content differed up to 74-fold between species and within species between cultivars.

With increasing extreme events in the course of climate change it can be expected that amount and allergenicity of pollen emitted change. The aim of the second study by Jung et al. (2021a) was therefore to investigate the impact of elevated air temperature and drought on flowering phenology of grasses and pollen characteristics. For this purpose, various cultivars from the annual grass species perennial ryegrass and timothy were exposed to different environmental conditions prior to flowering. The altered environmental conditions included drought and an increase in air temperature roughly one month prior to flowering in May to June. In particular, during drought, water deprivation resulted in a loss in plant vitality. While the perennial ryegrass grass species showed an increase in allergen content, the timothy species exhibited a reduction in allergen content.

Grasses are found almost everywhere, on large areas in agriculture but also on countless small marginal areas. Their actual influence on the pollen concentration is still relatively little studied and is therefore the aim of the third study by Jung et al. (2022). For this purpose, the 2015 daily pollen concentrations of the electronic Pollen Information Network (ePIN) measured at 25

locations in Bavaria were used. Land use data on grasses were extracted from InVeKos database for agricultural areas and from OpenStreetMap for all other areas. Concomitantly, cultivation intensity based on InVeKos and wind direction based on data from the German Meteorological Service (DWD) were used. The influence of land use on pollen concentrations was particularly strong in the first 10 km around the pollen stations and decreased steadily thereafter.

Environmental pollen studies in the field over several weeks are often affected by weather-related factors. Therefore, the fourth study by Jung et al. (2021b) investigated whether the twig method in climate chambers, which has already been used for phenology studies, is also suitable for pollen studies. The tree and shrub species studied were hazel, birch, and alder. For this purpose, the twigs with inflorescences were cut before the actual flowering and brought into a climate chamber for maturation and flowering. The pollen characteristics allergen content, protein content, pollen weight and pollen production were analyzed in the laboratory. The timing of cutting the twigs before natural flowering in the field followed by incubation under warmer conditions in the climate chamber influenced the timing of flowering and the characteristics of the pollen. It was shown that the twig method in climate chambers is suitable in principle for pollen studies including the possibility for climate manipulations.

This work has investigated some of the most important factors influencing pollen characteristics and pollen concentration. From a purely allergological point of view, timothy should be avoided in cultivation due to its high allergen content. On the other hand, it has been demonstrated that with increasing extreme events such as drought, the allergen content of timothy decreases. Thus, a precise consideration has to be made for future adapted seed mixtures.

For pollen concentration, it was possible to identify the distance of the source areas with the potentially highest influence. Further investigation is needed to determine which type of grassland use emits most pollen. The results on incubation under warmer conditions with changes in pollen characteristics could show, the twig method in climate chambers is a promising method for pollen research to perform climate manipulation experiments.

Zusammenfassung

Pollenallergien sind eine weit verbreitete Krankheit, und im Zusammenhang mit dem Klimawandel ist mit einer Zunahme der Prävalenz und der allergischen Symptome zu rechnen. Unter den windbestäubten Pflanzenarten spielen die Baumarten Birke und Erle, die Strauchart Hasel und die Gräserfamilie eine zentrale Rolle bei Allergien.

In dieser Dissertation wurden allergene Pollen in verschiedenen Feldexperimenten und durch die Analyse vorhandener Daten im Zusammenhang mit dem Landwirtschaftsmanagement und dem Klimawandel untersucht. Der Schwerpunkt lag dabei auf Gräsern, insbesondere der Familie der *Poaceae*, aufgrund ihres hohen allergenen Potenzials und ihrer ubiquitären Verbreitung. Die Ergebnisse wurden in vier Publikationen veröffentlicht.

Die Familie der Gräser zeichnet sich durch eine große Anzahl von Arten aus, die in der Landwirtschaft in zahlreiche Sorten unterteilt werden, deren Unterschiede im Allergengehalt noch relativ wenig untersucht sind. Daher wurden in der ersten Studie von Jung et al. (2018) sieben Grasarten und 15 Sorten untersucht und verglichen. Ziel war es, festzustellen, inwieweit sich die in der Landwirtschaft häufig angebauten Arten und ihre Sorten in ihren Polleneigenschaften unterscheiden. Die Laboranalyse konzentrierte sich in erster Linie auf die Bestimmung des Allergengehalts. Darüber hinaus wurden Proteingehalt, Pollengewicht und Pollenproduktion quantifiziert. Der Allergengehalt unterschied sich bis zu 74-fach zwischen den Arten und innerhalb der Arten zwischen den Sorten.

Mit zunehmenden Extremereignissen im Zuge des Klimawandels ist zu erwarten, dass sich Menge und Allergenität des ausgestoßenen Pollens verändern. Ziel der zweiten Studie von Jung et al. (2021a) war es daher, die Auswirkungen von erhöhter Lufttemperatur und Trockenheit auf die Blühphänologie von Gräsern und Pollenmerkmale zu untersuchen. Zu diesem Zweck wurden verschiedene Sorten der einjährigen Grasarten Deutsches Weidelgras und Lieschgras vor der Blüte unterschiedlichen Umweltbedingungen ausgesetzt. Zu den veränderten Umweltbedingungen gehörten Trockenheit und ein Anstieg der Lufttemperatur etwa einen Monat vor der Blüte im Mai bis Juni. Insbesondere während der Trockenheit führte der Wasserentzug zu einem Verlust der Pflanzenvitalität. Während die Deutsches Weidelgrasarten einen Anstieg des Allergengehalts aufwiesen, zeigte sich bei den Lieschgrasarten ein Rückgang des Allergengehalts.

Gräser sind fast überall zu finden, auf großen Flächen in der Landwirtschaft, aber auch auf unzähligen kleinen Randflächen. Ihr tatsächlicher Einfluss auf die Pollenkonzentration ist noch relativ wenig erforscht und ist daher das Ziel der dritten Studie von Jung et al. (2022). Hierfür wurden die 2015 an 25 Standorten in Bayern gemessenen Tagespollenkonzentrationen des elektronischen Polleninformationsnetzes (ePIN) verwendet. Landnutzungsdaten zu Gräsern wurden für landwirtschaftliche Flächen aus der InVeKos-Datenbank und für alle anderen Flächen aus OpenStreetMap extrahiert. Gleichzeitig wurden die Bewirtschaftungsintensität auf der Grundlage von InVeKos und die Windrichtung auf der Grundlage von Daten des Deutschen Wetterdienstes (DWD) verwendet. Der Einfluss der Landnutzung auf die Pollenkonzentration war in den ersten 10 km um die Pollenflugstationen besonders stark und nahm danach stetig ab. Umweltpollenuntersuchungen im Feld über mehrere Wochen werden häufig durch wetterbedingte Faktoren beeinflusst. Daher wurde in der vierten Studie von Jung et al. (2021b) untersucht, ob die Zweigmethode in Klimakammern, die bereits für phänologische Studien verwendet wurde, auch für Pollenstudien geeignet ist. Die untersuchten Baum- und Straucharten waren Hasel, Birke und Erle. Zu diesem Zweck wurden die Zweige mit Blütenständen vor der eigentlichen Blüte abgeschnitten und zur Reifung und Blüte in eine Klimakammer gebracht. Die Pollenmerkmale Allergengehalt, Proteingehalt, Pollengewicht und Pollenproduktion wurden im Labor analysiert. Der Zeitpunkt des Abschneidens der Zweige vor der natürlichen Blüte auf dem Feld und die anschließende Bebrütung unter wärmeren Bedingungen in der Klimakammer beeinflussten den Zeitpunkt der Blüte und die Eigenschaften der Pollen. Es wurde gezeigt, dass die Zweigmethode in der Klimakammer prinzipiell für Pollenstudien geeignet ist, einschließlich der Möglichkeit, das Klima zu manipulieren.

Im Rahmen dieser Arbeit wurden einige der wichtigsten Faktoren untersucht, die die Pollenmerkmale und die Pollenkonzentration beeinflussen. Aus rein allergologischer Sicht sollte Lieschgras aufgrund seines hohen Allergengehalts im Anbau vermieden werden. Andererseits hat sich gezeigt, dass mit zunehmenden Extremereignissen wie Trockenheit der Allergengehalt von Lieschgras abnimmt. Für zukünftige angepasste Saatgutmischungen muss daher eine genaue Abwägung vorgenommen werden.

Für die Pollenkonzentration konnte die Entfernung der Quellgebiete mit dem potenziell höchsten Einfluss ermittelt werden. Weitere Untersuchungen sind erforderlich, um festzustellen, welche Art der Grünlandnutzung die meisten Pollen emittiert. Die Ergebnisse zur Inkubation unter wärmeren Bedingungen mit veränderten Pollenmerkmalen konnten zeigen, dass die Zweigmethode in Klimakammern eine vielversprechende Methode für die Pollenforschung ist, um Versuche zur Klimamanipulation durchzuführen.

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Acronyms

AES = Agri-Environment Schemes

API = Annual Pollen Integral

BCA = Bicinchoninic Acid

BBCH = Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und Chemische Industrie (Federal Biological Research Centre for Agriculture and Forestry, Federal Plant Variety Office and Chemical Industry)

CI = Cultivation intensity (abbreviation only used in formulars)

CRM = Concentric ring method

DWD = Deutscher Wetterdienst (German Meteorological Service)

ePIN = Elektronisches Polleninformationsnetzwerk (electronic Pollen Information Network)

HFF = Hauptfutterfläche (Main feed area)

LfL = Landesanstalt für Landwirtschaft (Bavarian State Institute for Agriculture)

PBS = Phosphate-buffered saline

PP = Pollen peak

PSA = Potential source area

KULAP = Kulturlandschaftsprogramm (Bavarian Cultural Landscape Program)

RCP = Representative Concentration Pathways

RGV = Raufutter verzehrende Großvieheinheit (fodder-consuming livestock unit)

TBS-T = Tris-buffered saline with Tween20

VNP = Vertragsnaturschutzprogramm (Bavarian Contractual Nature Conservation Program)

1. Introduction

1.1 Allergies induced by pollen from wind-pollinated species

This work deals with various factors influencing the production and dispersal of pollen from wind pollinated plant species as well as the methodology of their analysis. An overall impression of the various influencing factors is given in Figure 1. After a general overview, the introduction is subdivided into the four studies conducted.

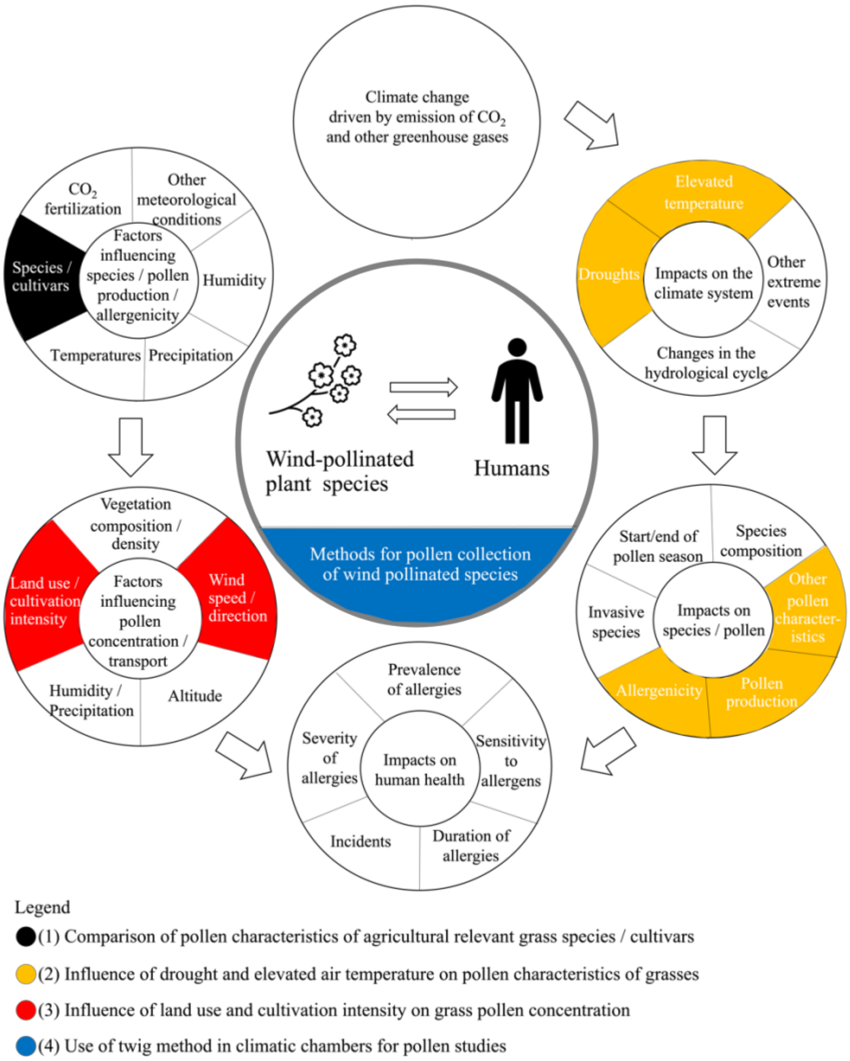

Figure 1 Overview of factors influencing the production, characteristics, and transport of pollen from wind-pollinated plant species, incorporating climate change, and the resulting implications for pollen allergy sufferers. The colors represent the addressed aspects of the individual studies (1), (2), (3) and (4). Figure content is based on Sofiev & Bergmann (2013), Beggs (2004) and Behrendt & Ring (2012).

Allergies caused by pollen account for the largest proportion of allergies in Europe (Burbach et al. 2009). In Germany, 14.8% of the population is affected by pollen allergies (Robert-Koch-Institut. 2015). Hay fever is a hypersensitive reaction of the body's immune system to plant pollen, mostly from wind-pollinators (Blackley 1873; D'Amato et al. 2007; Weger et al. 2013). There are a large number of different wind-pollinated species with varying allergenic potential (Table 1). In Europe, pollen from birch (*Betula*), grasses (*Poaceae*), ragweed (*Ambrosia*) and cypress (*Cupressus*) are predominant and have the highest clinical relevance (White & Bernstein 2003; Haftenberger et al. 2013; D'Amato et al. 2007; Smith et al. 2014).

Table 1 Overview of the most important wind-pollinated species occurring in Europe and the respective allergenic potential of their pollen in alphabetical order. The color of the fields indicates the respective allergenic potential of the pollen. The classification was made according to Saloga et al. (2012).

Artemisia *(Artemisia spp.)*	Chestnut *(Castanea spp.)*	Hornbeam *(Carpinus spp.)*	Parsley *(Apiaceae)*	Sorrel *(Rumex spp.)*
Alder *(Alnus spp.)*	Cichorioideae *(Cichorioideae)*	Larch *(Larix)*	Pine *(Pinus)*	Spruce *(Picea)*
Ash *(Fraxinus spp.)*	Composite *(Asteroideae)*	Legume *(Fabaceae)*	Plane *(Platanus spp.)*	Syringa *(Syringa spp.)*
Aspen *(Populus spp.)*	Chenopod *(Chenopodiaceae)*	Linden *(Tilia spp.)*	Plantain *(Plantago spp.)*	Taxus *(Taxus spp.)*
Bayberry *(Myricaceae)*	Cypress *(Cupressaceae)*	Maple *(Acer spp.)*	Poppy *(Papaveraceae)*	Thalictrum *(Thalictrum spp.)*
Beech *(Fagus spp.)*	Elm *(Ulmus spp.)*	Mulberry *(Moraceae)*	Privet *(Ligustrum spp.)*	Tsuga *(Tsuga spp.)*
Birch *(Betula spp.)*	Grasses *(Poaceae)*	Myrtle *(Myrtaceae)*	Ragweed *(Ambrosia spp.)*	Walnut *(Juglans spp.)*
Box *(Buxus spp.)*	Hazel *(Corylus spp.)*	Nettle *(Urticaceae)*	Rose *(Rosaceae)*	Willow *(Salix spp.)*
Cabbage *(Brassicaceae)*	Hemp *(Cannabaceae)*	Oak *(Quercus spp.)*	Rush *(Juncaceae)*	
Cattail *(Typhaceae)*	Heath *(Ericaceae)*	Olive *(Olea spp.)*	Sedge *(Cyperaceae)*	

Allergenic level	No	Low	Moderate	High

Allergic reactions result from frequent contact with allergens, during which the immune system becomes increasingly sensitized, especially in genetically predisposed individuals. The allergic reaction is caused by an allergen hitting the mucous membranes (eyes, nose or mouth). Symptoms of the allergic reaction typically include burning eyes and sneezing fits, and one in three hay fever patients also develops asthma attacks (bronchial) (Averbeck et al. 2007).

The severity of symptoms for allergy sufferers is highly dependent on the perceived pollen concentration, with symptoms increasing as pollen concentration increases (Durham et al. 2014; Weger et al. 2011; Caillaud et al. 2012). Pollen concentration in turn depends - in addition to weather, pollen transport, vegetation density, CO_2 fertilization effect and other factors - to a large extent on the flowering time of the individual species (Figure 2).

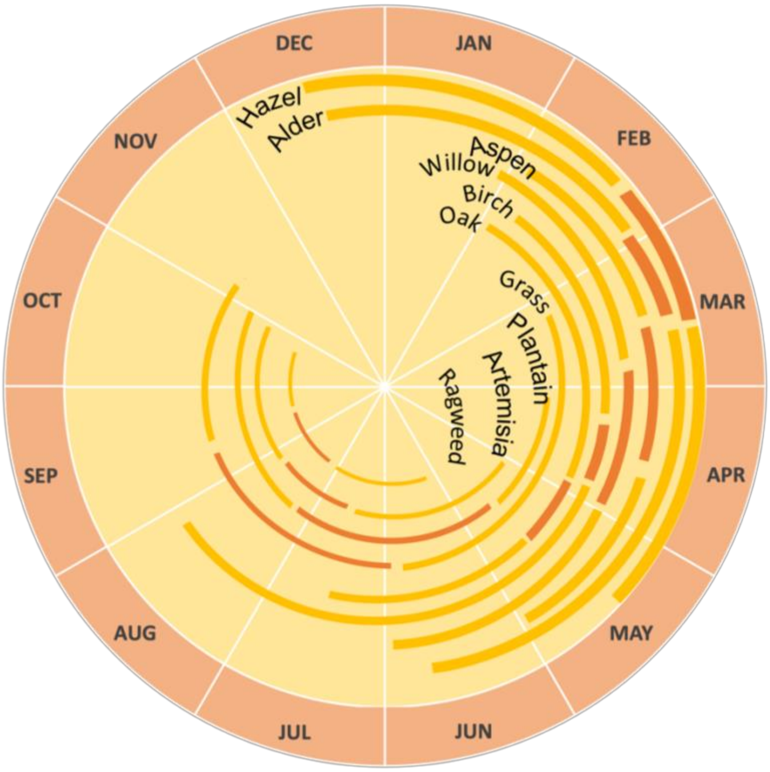

Figure 2 Pollen season of plant species with medium to high allergenic potential for the area of Germany. The main pollen season is marked in orange, possible further occurrences before and after the main pollen season are marked in yellow. Figure based on Apa pollenwarndienst (2021) and Stiftung Deutscher Polleninformationsdienst (2018).

As can be seen from this overview, there is a variety of factors that influence pollen production, characteristics, and transport of pollen from wind-pollinated plant species (see also Figure 1). Of these, several aspects were selected and investigated in the following four studies: (1) Comparison of pollen characteristics of agricultural relevant grass species / cultivars; (2) Influence of drought and elevated air temperature on pollen characteristics of grasses; (3) Influence of land use and cultivation intensity on grass pollen concentration; (4) Use of twig method in climatic chambers for pollen studies. The four studies are addressed in the following sections in turn.

1.2 Comparison of pollen characteristics of agricultural relevant species / cultivars

The first study was to investigate the extent to which different grass species and cultivars differ in their pollen characteristics and pollen production (1) (Figure 1). Knowledge gaps exist in that while information already exists on allergen levels for some of the most common grass species, this is not the case for grass species used in agriculture, particularly for cultivars. In this study, among other pollen characteristics such as pollen weight and protein content, group 5 allergen content was determined and compared in different grass species and cultivars using ELISA-assay. In general, the grass family (*Poaceae*) includes a huge number (~9000) of different species worldwide, most of which are wind-pollinated (Lewis et al. 1983; Andersson & Lidholm 2003). Along with this, it could be already shown that there are great differences between the individual species in terms of their flowering and pollen characteristics (Table 2). For southern Spain, it was shown that the main flowering period of grasses extends over a long period from April to mid-July, further extended by pre- and post-blooming periods (García-Mozo et al. 2010), demonstrating the high number of grass species. The amount of pollen released per inflorescence in each species varies greatly. In general, grass pollen is microscopic (Figure 3), with a wide range of particle sizes from 20 to 55 µm (Andersen 1979). As a consequence, the weight per pollen grain can also vary greatly across species (Table 2).

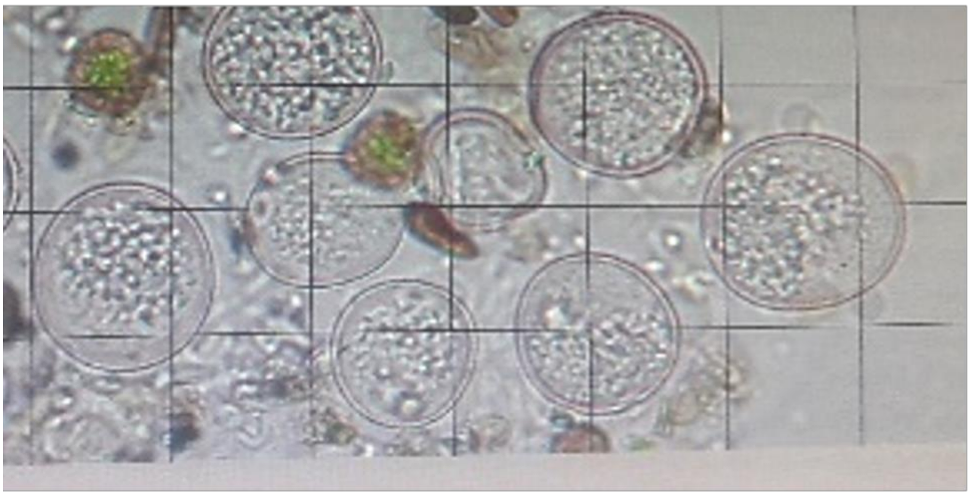

Figure 3 Grass pollen photographed under a transmitted light microscope. The size of grass pollen can vary from 20 to 55 µm. Photo by M. Stiel (2016).

Regarding allergies, the grain sizes of grass pollen are mostly not small enough to penetrate directly into the lower respiratory tract and thus directly trigger respiratory allergies. On the other hand, when the pollen is dissolved and its allergenic content is released, these components are small enough (0.6-2.5 µm) to cause allergic reactions in the distal part of the lungs as well (Suphioglu et al. 1992; Wilson et al. 1973).
Grass pollen is characterized by high sensitivity rates; according to Schmitz et al. (2013) 22.7% of the population is sensitive to timothy (*Phleum pratense* L.) grass pollen, what makes their precise research so important.

Table 2 Overview of the most important pollen characteristics for clinically particularly relevant grass species. Data were taken from Schäppi et al. (1999).

	Soluble group 5 allergen content (mg/mL)	Mass per pollen grain (ng/grain)	Soluble protein per pollen grain (ng/grain)	Soluble group 5 allergens per pollen grain (ng/grain)	Soluble protein per total grain mass (%)	Soluble group 5 allergens per total grain mass (%)	Soluble group 5 allergens per total soluble protein (%)
Perennial ryegrass	485	6.7	0.18	0.03	2.7	0.5	18.3
Italian ryegrass	584	8.9	0.19	0.05	2.1	0.6	28.2
Timothy	327	9.4	0.24	0.03	2.6	0.3	12.7
Orchard grass	424	11.4	0.32	0.05	2.8	0.4	15.1
Velvet grass	240	5.9	0.17	0.02	2.9	0.2	8.4
Fescue	192	10.8	0.27	0.02	2.5	0.2	7.7
Cultivated rye	491	23.8	0.61	0.12	2.6	0.5	19.2
Average	392	11.0	0.28	0.05	2.6	0.4	15.7
White birch (negative control)	n.d.[1]	18.8	0.28	0	1.5	0	0

[1]n.d. = not detectable

Thirteen different types of allergens can be found in grass pollen (Hrabina et al. 2008; Andersson & Lidholm 2003). The allergens from timothy are mostly cross-reactive and can therefore be found in numerous grass species. Among these 13 allergens, group 1 (glycoproteins, molecular mass of 32-35 kDa) and group 5 (glycoproteins molecular mass 28-32 kDa) allergens are the most relevant groups (Table 3), as up to 95% of patients have specific IgE for group 1 allergens and 80% for group 5 allergens (Hrabina et al. 2008; Esch 2008; Heinzerling et al. 2009; Sastre et al. 2015; Sekerkova et al. 2012; Marcucci et al. 2012; Duffort et al. 2008).

Table 3 Identification of the most allergologically relevant allergens in in the grass species timothy (*Phleum pratense* L.), which is used as a reference in studies on grass allergies. Table based on Andersson and Lidholm (2003).

Allergen	Molecular weight (MW), kDa (exp.)	Molecular weight (MW), kDa (calc.)	Isoelectric point (pI), (exp.)	Isoelectric point (pI), (calc.)	Function or similarity
Phl p 1	33-37	26.2	6.2-8.5	6.16	ß-expansin
Phl p 5	29-38	-	4.2-7.5	-	-
Phl p 5a	-	28.7	-	6.89	-
Phl p 5b	-	26.1	-	5.99	-

Group 1 and group 5 allergens are found exclusively in the subfamily *Pooideae* (Smith et al. 1994; Sharma et al. 2017) and show high homology in different grass species, but in group 1 allergens the immunodominant positions of amino acids may be different (Petersen et al. 1995). Therefore, the immune response to group 1 allergens may depend on the grass species. In comparison, the position of the immunodominant positions in group 5 allergen are rather constant. Therefore, group 5 allergens were used in this study, as in other studies for allergen quantification, because they are cross-reactive and can be detected equally in different grass species. In allergen determination, the timothy species serves as a reference, after which, for example, group 1 allergens are abbreviated as Phl p 1 and group 5 allergens are abbreviated as Phl p 5.

1.3 Influence of drought and elevated air temperature on pollen characteristics of grasses

The first study was about the pollen characteristics of agricultural species and cultivars. The second study was designed to build on the first study by examining how pollen characteristics and grass production change with climate change as air temperature rises and extreme events in the form of drought increase (2) (Figure 1).

On a long-term scale, climate change can lead to significant changes in phenology (van Vliet et al. 2002; Anderegg et al. 2021) and species composition, among other aspects (Barnes 2018; van Vliet et al. 2002). As a consequence of climate change, higher pollen concentrations and longer pollen seasons occur, resulting in significantly higher sensitization of the population to pollen (Lake et al. 2017). In particular, the meteorological conditions in the early flowering season have a decisive influence on the duration and intensity of the annual pollen season (Kurganskiy et al. 2021). Mild temperatures can lead to an earlier start of the pollen season (Picornell et al. 2019; Rojo et al. 2021; Lind et al. 2016) and prolong it, thus extending the period of pollen allergies (Anderegg et al. 2021). For instance, increases in spring temperature lead to a significant advance of budding and flowering (Fu et al. 2012; Wang et al. 2020; Menzel et al. 2006; Menzel et al. 2020b). In comparison, the end of the pollen season seems to be less affected and remains rather unchanged (Menzel et al. 2021). The question is how the group of grass species and cultivars respond to higher air temperatures and how the phenology of the species changes.

Changing growing conditions alter the distribution area of species, which leads to a shift in species composition (Hanna et al. 2018). For instance, the species *Ambrosia artemisiifolia* with very allergenic pollen, which was introduced to Europe by humans in the 19th century, will expand significantly more in the next decades due to changed climate conditions (Case & Stinson 2018; Cunze et al. 2013). With increasing warming due to climate change, the possibility of species migrating from warmer regions increases and the allergenic risk changes due to the shift in species composition for patients with pollen allergy (Lososová et al. 2018; Millennium ecosystem assessment, M. E. A. 2005).

In addition to changing species composition, the effects of climate change may also result in changes in locally present species, such as pollen characteristics and pollen production (Reinmuth-Selzle et al. 2017), which are the focus of the second study. It is expected that prolonged droughts with concurrent high air temperature are expected to increase sharply as climate change progresses (Dai 2013; Christensen & Christensen 2007) and, on the other hand, that extreme events with flooding will increase (D'Amato et al. 2020), both with significant consequences for human health (Zacharias & Koppe 2015). As a consequence from drought, heat or flooding, plants will experience stress, and this could also affect pollen. Extreme droughts are known to inhibit growth and limit pollen production, though the allergenic potential of pollen may increase due to plant stress (González Minero et al. 1998). A critical factor is the timing during plant growth when water shortage occurs (Craine et al., 2010). Compared to other taxa, herbaceous taxa are especially sensitive to water availability (Matyasovszky et al. 2015). For grasses, limitations in water availability during growth may affect the number of inflorescences (Craine et al. 2010). It was shown for Switzerland that dry and hot conditions in spring and summer led to earlier peaks in grass pollen, while the overall pollen season was shortened, and pollen production stopped at an early stage (Gehrig R. 2006). A grass species and cultivar-dependent response to elevated air temperatures with or without the inclusion of drought has been little studied. In particular, this study investigates whether elevated air temperatures and drought reduce or increase the allergen content of grass pollen.

With regard to pollen production, increasing CO_2 concentrations can have a direct influence on plants: The plants grow faster, become larger and more pollen is produced (Ziello et al. 2012; Beggs 2015). Among other reasons, and depending on the species, this can lead to higher pollen concentrations in the air, and thus higher exposure to allergenic pollen. Besides, elevated CO_2 concentrations can also cause the allergen content of pollen to increase in some species (Kim et al. 2018; Albertine et al. 2014).

1.4 Impact of land use on grass pollen concentrations

The previous studies were primarily concerned with the factors influencing pollen characteristics. In the third study, the focus is now on the factors influencing the pollen concentration in the air (3) (Figure 1). In general, meteorological conditions such as air temperature, precipitation, humidity, and wind speed/direction are decisive for pollen production and pollen dispersal (Schwartz 2013; Rojo et al. 2021). In wet conditions with high humidity, pollen cannot be dispersed and is washed out (American Academy of Allergy Asthma & Immunology 2022), whereas dry and warm conditions can facilitate pollen dispersal, increasing pollen concentrations in the air. Besides these meteorological factors, the concentration of grass pollen and its species composition in the air depends on the source areas and the geographical distribution (Emberlin & Norris-Hill 1991; Rojo et al. 2020). In relation to source areas and geographic distribution, land use plays an important role, which is characterized by the fact that it can change over time (Dale 1997; Emberlin 1994). As in most parts of Europe, Germany is strongly influenced by humans. The largest part is agricultural and forestry land (87%), followed by built-up land (9%) and only a very small proportion of completely unused land (<1 %) (European Environment Agency. 2012). In general, the potential release area for grass pollen is much smaller in urban areas than in rural areas (Kasprzyk 2006; Bosch-Cano et al. 2011; Majkowska-Wojciechowska et al. 2007). Especially in urban areas, grass-covered areas can be very small in size, making it extremely difficult to determine the impact of land use on pollen concentrations. For agricultural grasslands, which represent the largest area of potential grass pollen release, a general classification can be made. It can be divided into three basic types: arable land with cereal crops, pastures, or meadows (Fath & Jørgensen 2008). On agricultural grasslands, the number of cultivated species is significantly reduced in comparison to uncultivated grasslands. The arable land with cereal cultivation is clearly dominated by the cultivation of *Zea mays*. In permanent meadows and pastures in Germany, there are about 20 grass species with higher cover. In Bavaria, for example, the following species are the most common ones: *Lolium perenne* L., *Lolium multiflorum* Lam., *Dactylis glomerata* L., *Poa pratensis* L., *Poa trivialis* L., *Alopecurus pratensis* L., *Festuca pratensis* Huds., *Phleum pratense* L., *Trisetum flavescens* (L.) P.Beauv., *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl, *Festuca rubra* L., *Cynosurus cristatus* L., *Agropyron repens* (L.) Gould, *Agrostis spp.* L., *Holcus lanatus* L., *Bromus hordeaceus/mollis* L., and *Deschampsia caespitosa* P.Beauv. (Diepolder & Raschbacher 2007). Species are expanded and grassland complexity increases as species are subdivided into a large number of different cultivars (Bundessortenamt Hannover. 2021). For example, there are about 300 different cultivars of ryegrass (*Lolium perenne*) in Germany. These cultivars have been bred for specific site conditions and can, for example, increase yields or biomass.

Overall surveys of land use that include both, agricultural and non-agricultural grassland, have been limited. The first task of this study was to identify and classify all land uses with grasses and potential pollen release and to summarize them for the area of Bavaria.
Besides the actual land use, land management is an important factor influencing pollen concentrations, since for example, mowing hay or cutting silage can occur before most grass species reach the flowering stage, leading to significantly reduced emission of pollen (Menzel 2019). This can be counteracted by an earlier onset of the flowering period due to climate change, which leads to a greater time gap between the start of flowering and harvesting, unless the harvesting time is adjusted. Arguments against bringing the harvest date forward are, on the one hand, the historically established harvest dates and, on the other hand, the financial subsidies that farmers receive within the framework of Agri-Environment Schemes (AES) if they comply with certain measures (Batáry et al. 2015). The latter subsidies are incorporated, e.g. for Bavaria, Germany, in the Bavarian Cultural Landscape Program (KULAP) and the Bavarian Contractual Nature Conservation Program (VNP). Among other requirements for agriculture, these programs set a limit on the number of cuts per year, strictly regulate the timing of the first cut, specify the planting of sensitive areas with grassland, and determine the non-use of areas in the biotope category of meadows. These measures would basically result in more grass species being able to flower and consequently more pollen being released.
However, the fact that hay production was largely replaced by silage production argues against an increased release of grass pollen. For the production of silage, it is very important that the cutting date occurs at the time when the ear emergence of the first species in the sward (field foxtail (*Alopecurus pratensis*) begins, because the protein content and thus the fodder quality decreases immediately afterwards (Kuoppala et al. 2008). Silage production has also greatly increased the average number of cuts per year in recent decades and can result in up to six cuts per year. It can be assumed that already with two cuts of the grassland per year, flowering of most grass species must be ruled out (Skjøth et al. 2013). Reducing the number of cuts per year also increases the chance that grasses will come into flowering. The timing of the first cut is absolutely critical, as most grasses produce significantly less pollen at the second flowering or do not flower a second time at all. In the specific case of pastures, the number of animals per unit area, the type of animals, and the duration of grazing are the most important factors determining potential pollen release (Groenman-van Waateringe 1993).

In forests, which in the example of Bavaria account for about one-third of the area (Wiesmeier et al. 2013), grasses are most likely to be found only at the very edge, along forest roads, canopy openings or in areas of recent re- / afforestation. Unlike agricultural land, these grasses are not cut regularly, if at all. In the heavily built-up areas of cities, sources of pollen are found in much smaller areas such as gardens, parks or roadside greenery. There is a high range in the number of cuts here, from not at all to several times per month. On completely unused grasslands there is a high likelihood for pollen release because they are not cut at all or only from year to year. However, these grasslands are usually very small and difficult to identify compared to the other land uses. Information on the cultivation intensity depends to a large extent on the respective farmers, and has so far only been included in other studies to a limited extent. The objective of this study was to obtain information on cultivation intensity in the form of cutting frequencies via existing indicators and link them to the identified land uses and, in turn, correlate them to grass pollen concentrations.

In addition to source areas, cultivation intensity, and other factors, pollen concentration is determined by transport distance. Depending on the pollen's respective aerodynamic diameter, weight, and release height, the transport distance can vary greatly, ranging from a few meters to several 100 km (Smith et al. 2005). In the literature there are several definitions for a possible subdivision of the transport distance into short-, medium-, and long-distance transport. Commonly used is the approach proposed by Sofiev and colleagues (2013) with three different scales for pollen transport: Micro and local transport (meters to kilometers), regional transport (up to 100 km), and long-distance transport (>100 km). Local transport is driven by micrometeorological conditions and wind speed / direction (Di-Giovanni & Kevan 1991). In comparison, medium-range transport is closely linked to meteorology and climatology at the regional level (van de Water et al. 2003; Damialis et al. 2005) and long-distance atmospheric transport depends on meteorological conditions at the continental scale (Campbell et al. 1999). Birch and grass pollen have different preconditions for their dispersal. While birch pollen is largely released about 10 meters above the ground, grass pollen is released near the ground. A greater release height of bioaerosols, among others, generally reduces the concentration near the source but increases the dispersion area (Steinfeld 1998). Birch pollen, with a diameter of about 20 µm (Mäkelä 1996), is much smaller than most of the grass pollen (20 to 55 µm) (Owensby et al. 1970; Joly et al. 2007). These characteristics favor long-distance transport of birch pollen, whereas grass pollen is largely restricted to local and regional transport.

Pollen concentrations often decrease at higher elevations due to the diminishing land area, plus lack of suitable local growing conditions for the plants and less species, making many species less likely to be found over a large area. The effect of altitude on pollen concentration is strongly dependent on species as shown in a study of 24 species, where pollen concentration was reduced in only about half of the species (Damialis et al. 2017). Grasses are still widespread at altitudes of 1500 m above sea level, so that pollen concentrations remain relatively constant and only decrease at higher altitudes (Gehrig & Peeters 2000). This is important for the present study because pollen concentrations from different altitudes have been used.

Compared to grasses, birch pollen, for example, was found in larger quantities at higher elevations even without local occurrence (Gehrig & Peeters 2000). Here, the lack of pollen released by local vegetation can be at least partially compensated by pollen from other regions via medium and long distance transport which has relevant influence on the pollen concentration (Menzel et al. 2021; Frei 1997) In grasses, long-distance transport is rarely observed. Instead, it is limited to regional distances, so that relevant pollen concentrations are no further than 30 km from the source (Frisk et al. 2022). For this reason, the 30-km radius was included in terms of pollen concentration in this study. Wind direction and wind speed were included in the pollen concentration with respect to the transport distance.

1.5 Climate chambers for pollen investigations

The fourth study deals with the methods used in experiments for pollen studies. The aim was to experimentally investigate a method already known from phenology studies for its applicability to pollen studies on different tree and shrub species (4) (Figure 1). Birch pollen is one of the predominant tree pollen in Europe and is a major cause of pollen associated allergies (Smith et al. 2014). Among the wind-pollinated plants, its accurate study is therefore of great importance for allergy sufferers. Birch pollen is characterized by its ability to directly enter the lower respiratory tract and trigger asthma attacks due to its small pollen size (~22 µm). Of highest clinical relevance among the birch pollen allergens is Bet v 1 sensitization (Jarolim et al. 1989; Gao et al. 2021). A study for Germany could show that ~14% of the population is sensitized to Bet v 1 allergens (Schmitz et al. 2013). Crucially, other species of the *Betulaceae* and *Fagaceae* families (alder, hazel, oak, chestnut, hornbeam, and beech) show significant IgE cross-reactivity of allergen homologues with Bet v 1 (Biedermann et al. 2019; Niederberger et al. 1998; Weber 2003).

The examination of pollen, such as the determination of allergen Bet v 1 in various tree and shrub species, requires the collection of a sufficient amount of pollen, which is sometimes associated with high technical effort. For the collection of pollen there are technical solutions in form of high-volume impactors (Buters et al. 2010; Grewling et al. 2020) or low-volume devices such as multi-vial cyclone collectors (Brennan et al. 2019). These technical solutions offer the advantage that pollen samples can be collected relatively easily and protected from weather conditions, but they are associated with high acquisition costs and it is hardly possible to collect pollen from only one species. Other established methods of pollen collection are lacking.

For the collection of pollen there is another possibility to use special glassine bags, into which the pollen is directly emitted. This method is very cost-effective and pollen collection is also possible for individual species. However, a major drawback is that although the bags are permeable to air, at the same time they are very sensitive to moisture. Exposed to natural weather conditions, moisture can form in the bags, as a result of which mold forms and the pollen clumps. In addition, the bags can be damaged by insect infestation. The question arises as to how these disadvantages can be overcome in pollen collection in the field, while at the same time keeping costs low. A solution is proposed in this study by combining it with other already established methods.

The aim of this study is therefore to find out whether the twig method in climatic chambers, which has so far been used mainly for studies of phenology (Basler & Körner 2012; Dantec et al. 2014; Primack et al. 2015; Menzel et al. 2020a). In particular, it is tested whether twigs cut from the tree before flowering and then transferred to the climate chamber for maturation and provided with collection bags provide a sufficient amount of pollen for downstream investigations.

If the method is successful, climate chambers offer the advantage that potentially various climate scenarios can be run with regard to climate change. This has already been successfully demonstrated in various studies on phenology. For example, the influence of (chilling) temperatures (Laube et al. 2014a) or the development of buds at elevated humidity (Laube et al. 2014b) could be determined. Studies have for instance already shown that the response mode to changing climatic conditions strongly depends on the species (Laube et al. 2014a; Malyshev

2020; Miller-Rushing & Primack 2008). Therefore, this study aims to investigate for pollen studies whether outdoor climate conditions can be manipulated using climate chambers.

2 Outline

Airborne pollen concentrations and pollen characteristics are influenced by a variety of factors, including climatic factors, spatial distribution of species, and season. In the investigations within the scope of this work, the first study investigates the family of grasses in terms of pollen characteristics, with a special focus on allergen content. The second study also investigates pollen characteristics of grasses but considers climate change impacts. The third study examines the relationship between land use and pollen concentration. In the fourth study, the suitability of the twig method in climatic chambers for pollen studies is investigated. The studies include data generated from own experiments as well as on already existing data sets.

The following research questions were defined:

1. A major trigger of allergies are grass pollen, which have a high allergenic potential. Of the large family of grasses, only a very small proportion of species have been studied for allergenicity previously. Even less is known about the allergenicity of grass cultivars used in agriculture. Therefore, the following questions were addressed:

    - To what extent do grass species relevant in temperate agriculture differ in their pollen characteristics (allergenicity, protein content, pollen weight)? (section 5.1)

    - Are there differences in allergenicity between different cultivars within one grass species? (section 5.1)

2. It is known that climate change will influence species composition and distribution. One of the main drivers is the increase in air temperature. As a result of climate change, there may be an increase in extreme events such as floods or droughts. Relatively little is known about the effects of climate change including drought and elevated air temperature on the allergenic potential of grass pollen. Based on this, the following questions were addressed:

    - What influence do drought and elevated air temperature have on flowering timing, pollen concentration and pollen characteristics of grass pollen? (section 5.2)

- Do grass species differ in their pollen characteristics in the response to changing climatic conditions? (section 5.2)

3. The concentration of pollen in the air is directly related to the amount of pollen released, resuspended or washed-out and the distance over which additional non-local pollen are transported. The decisive factors are environmental conditions including temperature, precipitation, wind speed, and wind direction that can promote the release and the distribution, but also hinder it. In the case of grasses in particular, humans can have a significant influence on the release of pollen in the form of grass cutting. The following questions were addressed regarding pollen concentrations:

    - How large is the influence of land use on grass pollen concentration? (section 5.3)

    - Is the influence of land use reflected in a spatial change in pollen concentration? If yes, in which distance from the pollen source is the influence of land use on pollen concentration greatest? (section 5.3)

4. Pollen collections in the field are often confronted with problems due to weather conditions and a high organizational effort. In pollen studies, the step of collecting pollen in the field is particularly sensitive, as complete failures due to weather can happen quickly. So far, technical possibilities are restricted to expensive instruments by which selective pollen collection is hardly possible. The purpose of this work was to investigate the twig method as alternative method for the adequate collection of pollen. To this end, the following questions were posed:

    - Is the twig method in climatic chambers also suitable for the collection and examination of pollen? (section 5.4)

    - Can climate chambers be used as proxies for outdoor manipulations in pollen studies? (section 5.4)

3 Overview of data and methods

3.1 Study sites overview

First, a brief general overview of the study sites is provided, then the study sites are explained in more detail and the methods used for each study are presented.

The study sites for the experiment-based studies were located near the campus of the Technical University of Munich in Weihenstephan, Freising, Germany (Figure 4a). It was possible to build on earlier experimental setups, which were, if necessary redesigned, to the requirements of the present studies.

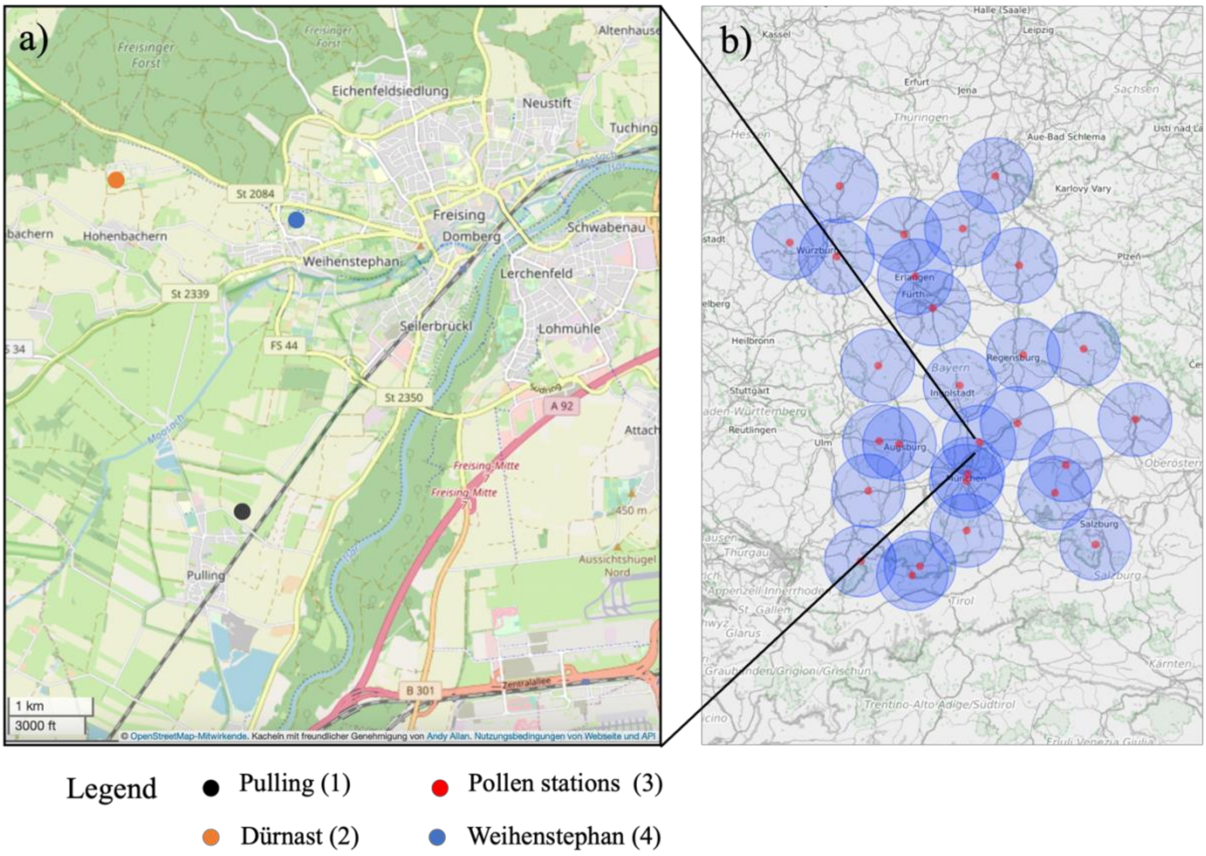

Figure 4 Overview of study sites: a) (1) Pollen characteristics of agriculturally relevant grass species / cultivars, Pulling; (2) Impact of drought / elevated air temperatures on grass pollen characteristics, Dürnast; (4) Twig method in climate chambers for pollen studies, Weihenstephan; b) (3) Influence of land use on grass pollen concentration, location of the 27 pollen stations in Bavaria, Germany, red dots mark the position of the pollen traps, the light blue circles indicate the 30 km surroundings.

3.2 Comparison of pollen characteristics of agricultural relevant species / cultivars

3.2.1 Study site and investigated species

The study site in Pulling (1) (Figure 4) (48.3712˚N, 11.7181˚E, 455 m a.s.l.) belongs to the Bavarian State Institute for Agriculture (LfL), where different grass species and cultivars from agricultural management were grown perennially for educational purposes (Figure 5). Different grass species and their cultivars were studied which were selected mainly on the basis of their importance in agricultural management. The main focus was on the species ryegrass and timothy.

Figure 5 Overview on parts of the study site in Pulling operated by the LfL. Different plots can be seen, on which various grass cultivars are grown in monoculture. Photo by E. Handelshauser (2019).

3.2.2 Phenological development according to BBCH

Phenological development of inflorescences of the studied plant species / specimens of grasses was documented at regular weekly intervals before and after cutting in the field. The main aim was to detect the moment when the flowering starts. The individual stages were recorded according to the respective species-specific key by the Federal Biological Research Centre for

Agriculture and Forestry, Federal Plant Variety Office and Chemical Industry (BBCH) (Meier 2001) (Figure 6).

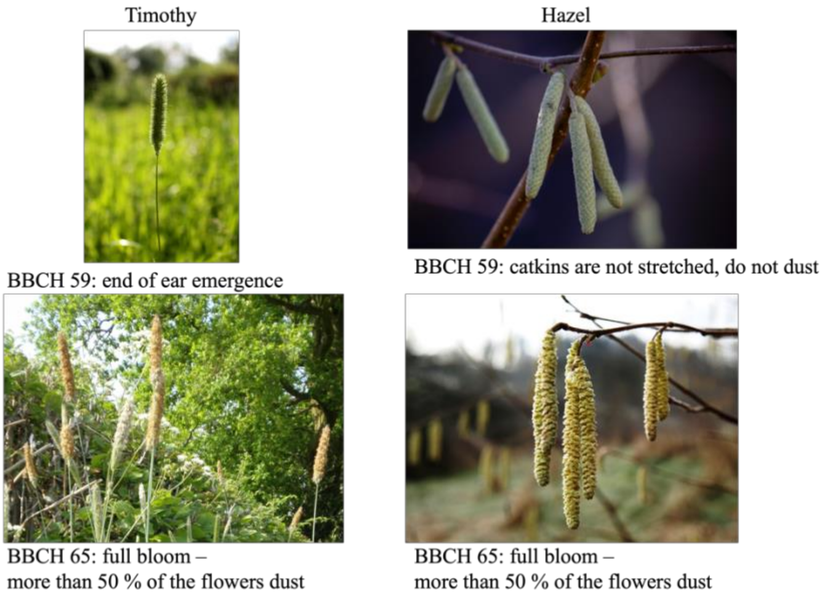

Figure 6 Phenological development of inflorescences according to the BBCH (Meier 2001) growth stages 59 and 65 for the plant species timothy and hazel. Image bottom left adapted from Amanda Slater / flickr.com / https://creativecommons.org/licenses/by-sa/2.0/, other images taken from Pxhere.com which are released under Creative Commons CC0

3.2.3 Experimental procedure

When first flowering appeared for a cultivar (BBCH 61), 10 to 20 plant individuals per plot were cut and inflorescences were put into pergamin bags. Pollen bags were stored under fixed conditions in the climate chamber (day-night cycle: 14 hours day at 23˚C, 10 hours night at 15˚C; air humidity 45%) until full flowering was reached. Pollen was separated from the anthers and was then stored at -20˚C until further laboratory analysis.

3.2.4 Laboratory analysis

During the separation of pollen, the grass culms were counted. The total amount was determined with an electronic balance (XS204DR, Mettler Toledo GmbH, Gießen, Germany). From the ratio pollen / number of culms the produced amount of pollen was calculated.

For the pollen extraction, 5 to 10 mg pollen (depending on the overall available pollen amount) was given into Phosphate-Buffered Saline (PBS) 20x and continuously shaken for several hours. The supernatant was then stored again at -20˚C.

The average pollen weight per pollen grain was calculated from the count of respectively 5 mg pollen in a cell counter (TC-10, Bio-Rad Laboratories GmbH, München, DEU). Each count was performed with four replicates, and the results were averaged.

Two different methods were used for the determination of total protein levels: Bicinchoninic Acid (BCA) Protein Assay and Bradford. Both methods were tested for the pollen species studied in a pre-test with purification of the samples and gel electrophorese. The results of the BCA analysis were in better agreement with other relevant studies compared with the Bradford analysis, in consequence BCA was applied for grass pollen samples. For the BCA assay, albumin from Serva (11930) was used as a standard with concentrations ranging from 25-1000 µg/ml, and BCA solution and copper sulfate were from Sigma (B9643; C2284).

The allergen group 5 quantification was conducted with a commercial sandwich ELISA kit (Allergopharma GmbH, Reinbeck/Hamburg, Germany) with a sensitivity of 1 ng/ml and a precision of ±10%. The allergen Phl p 5 from the grass species timothy served as reference in the test. A standard curve was created from the different concentrations of this allergen, covering a concentration range of 1 to 1000 ng/ml.

The two monoclonal antibodies MoAb 1D11 and MoAb B01 (Allergopharma GmbH) were applied to bind the present epitopes on the grass pollen allergens Phl p 5a and Phl p 5b. With these two antibodies together with a chromogen, epitope-antibody binding was detectable spectrophotometrically and allergen content could be quantified. Group 5 allergens occur as homologous proteins in soluble extracts from all grass species, therefore the MoAb 1D11 and MoAb B01 antibodies could be used to quantify allergens in all grass species.

3.3 Influence of drought and elevated air temperature on pollen characteristics of grasses

3.3.1 Study site and investigated species

The second experimental site (2) (Figure 4) dealing with grasses was located in Dürnast within the Dürnast Research Station (48.404457˚N, 11.690464˚E; 445 m a.s.l.), which has already been used for drought experiments on plants (Taeger et al. 2015). In one of the previous experiments, the loamier soil has been replaced with sandy material to better elicit effects of drought (Martínez-Sancho et al. 2017). Four different cultivars each of ryegrass and timothy were studied in this experiment. The grass cultivars were sown in the fall of 2016. Per plot, 0.54 g of pure seed of one cultivar was mixed with soybean grist and evenly distributed over the respective 65 x 50 cm area.

3.3.2 Experimental procedure

The impact of elevated air temperatures and drought on grasses and especially grass pollen was investigated in a full factorial treatment design including drought and elevated air temperature (Figure 7).

Figure 7 Overview of the experimental setup in Dürnast with the different treatments. On the left side: drought treatment (rear), warming + drought treatment combined (front). Right side: control (rear) treatment and warming treatment (front). Photo by S. Jung (2017).

Drought stress was implemented by a rainout shelter ('drought') and air temperature was elevated by a micro-capillary warm water system installed in 20 cm height ('warming'). Three replicates were created for each cultivar and treatment, resulting in 36 plots per treatment and a total number of 144 plots. The treatments were first applied when first grasses reached the height of 20 cm. In order to prevent shading effects, the warming system was installed just before the treatments started. The phenological growth stages were recorded following point 3.2.2. When first flowering appeared for a cultivar / treatment (BBCH 61), the plants were treated following point 3.2.3. The analysis in the laboratory to determine the pollen characteristics followed point 3.2.4.

Between May 19 and June 23, 2017, soil moisture was recorded two to three times per week at depths of 100, 200, and 300 mm using a soil moisture sensor (PR2 /6 SDI-12, HH2 Moisture Meter, Delta-T Devices, Cambridge, UK) at 36 locations evenly distributed throughout the plots. Due to very low soil water content, all plots were irrigated with a watering can at 1.6 L per plot on June 01 and 3 L per plot on June 09/14/21, 2017.

Meteorological data (air temperature, humidity, and precipitation) at hourly resolution were obtained from a nearby climate station (Weihenstephan-Dürnast, location 48.4029°N; 11.7305°E, distance to field site 385 m) of the DWD. In addition, air temperature and relative humidity were measured at 20 cm above the ground with 12 sensors directly at the site, evenly distributed among the treatments.

During the manipulation experiment with drought and warming, digital images were taken of each plot during the study period May 30 and June 12, 2017. The green values (DN, digital number) of RGB images (*.jpeg) were extracted with the package Fiji (Schindelin et al. 2012), which is based on ImageJ (Rueden et al. 2017). In the evaluation, the green values obtained were used as a proxy for plant vigor.

3.4 Influence of land use and cultivation intensity on grass pollen concentration

3.4.1 Pollen data from ePIN

In the measuring campaign in 2015 by ePIN network, daily pollen concentrations of 15 pollen species were recorded at 25 different sites in Bavaria, Germany (Oteros et al. 2019) (3) (Figure 4). In addition, two further measuring stations in Freising (own measurements) and Augsburg (operated by TUM/UNIKA-T) were used. For the pollen measurement the widely used methodology of Hirst traps was used (HIRST 1952) following guidelines of the European Aerobiology (Galán et al. 2014). From these, the station-specific parameters length of pollen season, day with highest pollen load, annual pollen integral (API) and pollen peak (PP) were calculated. API describes the sum of pollen concentrations during the pollen season and PP is defined as the highest daily pollen concentration.

3.4.2 Land use data from InVeKos, OpenStreetMap, RGV/ha HFF

Spatial information on agricultural land and land use in Bavaria was obtained from the InVeKos (Integrated Administration and Control System) database in cooperation with the LfL. All agricultural land with wild grass was extracted from this dataset. The grassland data obtained had high spatial resolution at the cadastral unit level. Additionally, the variable "RGV/ha HFF" which refers to the financial reward for complying with certain thresholds in agricultural management farms could be used to determine the cultivation intensities of the individual areas. "RGV/ha HFF" describes the number of cattle per hectare fodder (RGV = fodder-consuming livestock unit equivalent to 500 kg live weight) divided by the sustaining area (HFF = permanent grassland, silage maize and arable fodder). Besides InVeKos, spatial information on agricultural as well as non-agricultural grasslands was derived from OpenStreetMap. In order to determine the non-agricultural grasslands, the non-overlap between grasslands from InVeKos and OpenstreetMap was calculated.

Land use data from the InVeKos / OpenStreetMap databases were processed in ArcGIS version 10.6.1 as vector data in EPSG:32632 projection. 60 rings with corresponding buffers of 500 m for subsequent correlation between land use and pollen concentration were generated in ArcGIS.

3.4.3 Weather data (DWD)

The weather data of the DWD in hourly and in daily resolution were obtained from its server, extracting air temperature, precipitation, wind speed, and wind direction parameters. Of the available DWD stations, those closest to the study sites (distances could vary from hundreds of meters to around 40 km) were selected.

3.4.4 Data processing

The concentric ring method (CRM) by Oteros and collegues (2015) was used to study the relationship between the correlation of potential source area and pollen concentrations.

Here, the potential source area (PSA) for grassland was calculated as follows:

$$\mathrm{PSA} = \frac{\sum_{1}^{n} \mathrm{A}_{\mathrm{grassland,i,n}}}{\mathrm{A}_{\mathrm{ring,i}}}, \mathrm{i} = 1 \ldots 60$$

where A is the size of the area, i is the ith concentric ring from the center (pollen trap location) to the maximum 30-km distance per 500 m, and n is the nth polygon grassland area within the concentric ring.

In the following, the potential source area weighted by cultivation intensity (PSA_{CI}) was calculated:

$$PSA_{CI} = \frac{\sum_1^n \left[A_{grassland,i,n} / \left({CI_{i,n}}^3 + 1\right)\right]}{A_{ring,i}}, i = 1 \dots 60$$

where $CI_{i,n}$ is the cultivation intensity CI (RGV / ha HFF) for each grassland area. The CI weighting of grassland was done by summing the RGV / ha HFF weighted grassland areas for each ring and then dividing this sum by the whole ring area.

Finally, the potential source area, weighted by cultivation intensity and directional wind frequency, was calculated:

$$PSA_{CI+wind} = \frac{\sum_{i=1}^4 \left(\omega_i * PSA_{CI+wind,i}\right)}{\sum_{i=1}^4 \omega_i}$$

where ω_i is the wind frequency in the wind directions (NE, NW, SE, and SW) during the 2015 pollen season at the respective pollen stations. For weighting, each ring was divided into four quarters, resulting in a total of 240 subsections for each pollen station. For each ring subsection, the respective pollen source area was divided by the total area of the subsection.

3.5 Climate chambers for pollen investigations

3.5.1 Study site and investigated species

For the third experimental set-up in which tree and shrub species were studied, two birch, two alder, and three hazel specimens were selected within 500 meters of the campus Weihenstephan, Freising (48.400292 ˚N, 11.716874 ˚E) (4) (Figure 4 and Figure 8). The selected trees / shrubs were located in a minimum distance of 15 meters from buildings to avoid their influence. The selected hazels were approximately the same age (20 years), had the same height (8 m) and diameter (20 cm). The studied alders were probably of the same age (25 years), had the same height (15 m) and diameter (30 cm). The birch trees studied were of different age (30 and 50 years), height (15 and 25 m), and diameter (30 and 40 cm).

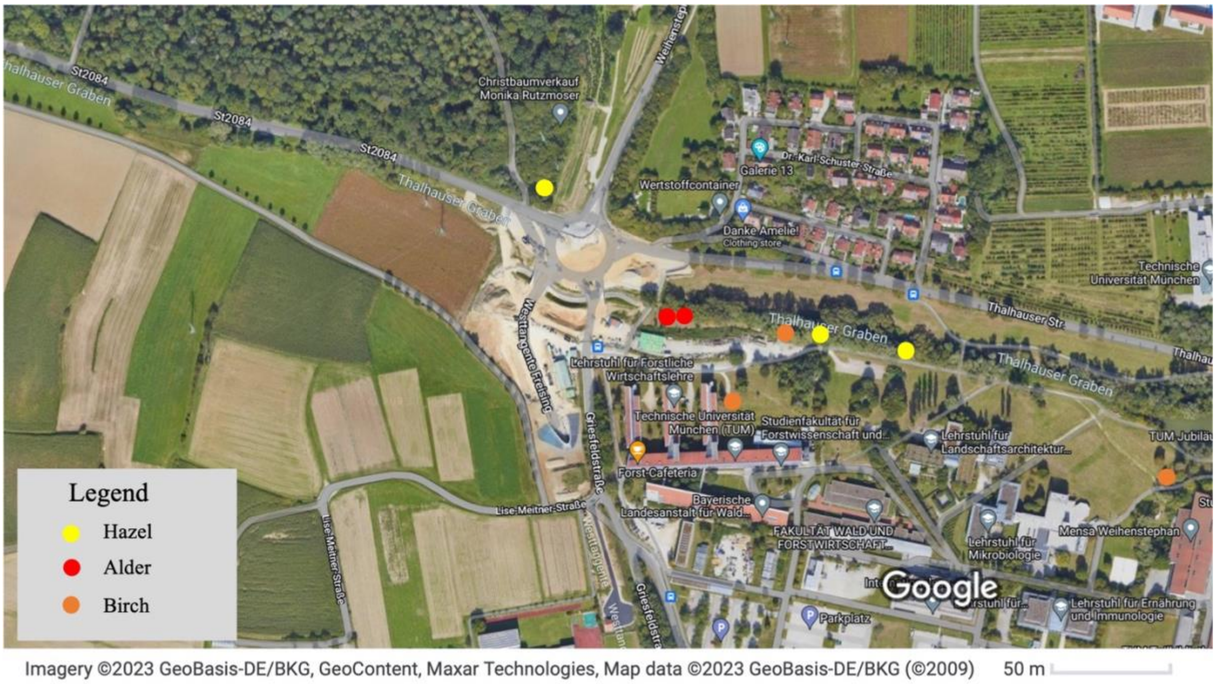

Figure 8 Map of the Weihenstephan campus, Freising with the selected specimens of hazel, alder and birch. Map is based on https://www.google.com/maps.

3.5.2 Experimental procedure

Twigs were cut from the donor trees in weekly intervals before actual flowering (maximum four weeks). Documenting the phenological development of inflorescences of the studied plant specimens of hazel, alder and birch followed point 3.2. For each species, substrate, and harvest date, an average of four twigs with at least five male inflorescences per twig were harvested. As in the drought experiment for grasses, the inflorescences were covered with glassine bags (Figure 9). The twigs were stored in glass bottles using up to three different substrates, namely pure water, tissue fertilizer and plant fertilizer. All twigs were treated with a remedy against mold growth. Until full flowering, twigs were stored under fixed conditions in the climate chamber (day-night cycle: 15 hours day at 20 °C, 9 hours night at 15 °C; air humidity 78%). The pollen was separated from the anthers and stored at -20˚C. Hobo loggers (type U23 Pro v2) were used to record air temperature and humidity in the field and in the climate chamber in the shortest possible distance from the samples.

Figure 9 Cut twigs where the inflorescences are covered with glassine bags and are stored in the climate chamber until flowering. Photo by S. Jung (2019).

In addition, comparisons were made between cut and uncut twigs under field conditions on the selected tree / shrub specimens (Figure 10). For this purpose, individual twigs were cut from the donor trees before natural flowering and reattached to the donor tree in plastic water containers. The same number of uncut twigs was selected as control. For in situ pollen collection, inflorescences were also covered with glassine bags just before flowering.

Figure 10 Comparison between cut and uncut twigs under field conditions on the tree / shrub just before flowering. Photo by S. Jung (2019).

3.5.3 Laboratory analysis

The laboratory analysis largely followed that for the grasses (3.2.4), with differences in protein and allergen determination.

As with the grasses, two tests were used to determine total protein content: BCA and Bradford. The Pierce BCA^{TM} Protein Assay was used for the BCA assay and the Bradford Bio-Rad Protein Assay Dye Reagent Concentrate was used for the Bradford assay. Bradford analysis for pollen samples from trees and shrubs were highly comparable to other similar studies, therefore Bradford was applied.

Allergens were detected using the Western Blot technique. In comparison to the ELISA-assay for grass pollen, generally all available allergens could be visualized in the Western Blot. After performing gel electrophoresis, Western blot comprised the addition of three antibodies in chronological order: human antibody (sera mixture of 29 patients allergic to Birch) (overnight exposure), monoclonal anti-human IgE antibody produced in mouse (Sigma-Aldrich) (two-hour exposure), and rat anti mouse IgG2b (produced by The Monoclonal Antibody Core Facility at the Helmholtz Center) (one-hour exposure), which were washed in between with Tris-buffered saline with Tween20 (TBS-T). The final detection of the allergens was made under chemiluminescence (ECL™ select Western blotting detection reagent). Signal intensity was extracted from chemiluminescence output using Fiji. The band for the allergen Bet v 1 was most distinct among the allergens. For the quantification of the allergen content, the total protein content was additionally determined. For this purpose, staining with Ponceau-S was performed during the Western blot before adding the first antibody, which was subsequently washed out by the addition of milk powder. The allergen content was derived from the division of the extracted chemiluminescence and Ponceau S staining values.

3.6 Statistical methods

All data were first tested for normal distribution using the Shapiro-Wilk test. This showed that the parameters studied were not normally distributed (p-value of the Shapiro-Wilk test < 0.05), due to which nonparametric tests were subsequently used. Specifically, Student's t-test was used for comparisons between two groups, univariate ANOVA (Kruskal-Wallis test) for comparisons with more than two groups followed by pairwise Wilcoxon test for comparison of individual groups. P-values of less than 0.05 were considered statistically significant.

3.7 R software

R software was used for data processing and analysis (R Core Team 2018). Among others, the packages dplyr (Wickham et al. 2018), latticeExtra (Sarkar & Andrews 2022), tidyr (Wickham & Girlich 2022) and ggpubr (Kassambara A 2020) were used. The R package AeRobiology (Rojo et al. 2019b) was used to calculate pollen indices. Data visualization was performed with the R package ggplot2 (Wickham 2016).

4 Publications: Summaries and contributions

This section contains the abstracts and author contributions of the four journal articles that were published as part of this work and are reprinted with permission from the respective journals in the appendix (open access under Creative Commons Attribution License). Paper 1 (Section 4.1) Comparison of pollen characteristics of agricultural relevant grass species / cultivars. Paper 2 (Section 4.2) Influence of drought and elevated air temperature on pollen characteristics of grasses. Paper 3 (Section 4.3) Influence of land use and cultivation intensity on grass pollen concentration. Paper 4 (Section 4.4) Use of twig method in climatic chambers for pollen studies. Abbreviations with initials of the first name and the family name are used in the author contributions (e.g. SJ for Stephan Jung).

4.1 Comparison of pollen characteristics of agricultural relevant grass species / cultivars

Jung, S., Estrella, N., Pfaffl, M. W., Hartmann, S., Handelshauser, E., & Menzel, A. (2018). Grass pollen production and group V allergen content of agriculturally relevant species and cultivars. *PLOS ONE*, *13*(3), e0193958. https://doi.org/10.1371/journal.pone.0193958, IF 3.5 (2021).

Abstract

Grass pollen is the main cause of hay fever and allergic asthma in warm temperate climates during summer. The aim of this study was to determine the content of group 5 major aller- gens in pollen grains of agriculturally important grass species/cultivars. For each cultivar flowering dates and pollen production of cut anthers were observed in the field and in a cli- mate chamber, respectively. An ELISA was used to quantify the group 5 allergens (Phl p 5) in pollen extracts which were gained from the grass species Kentucky bluegrass, perennial ryegrass, timothy, cocksfoot, annual / Italian ryegrass, hybrid ryegrass and festulolium. The group 5 allergen content of species varied between 0.01 ng (Kentucky bluegrass) and 0.06 ng (timothy) per pollen grain. On cultivar level the pollen allergenic content differed up to 74-times within the selected grass species. Results from this study might be helpful for the reduction of allergen exposure coming from agriculture grass production e.g. by an adapted grass selection or by the cultivation of grasses with low allergenic content in plant breeding.

Author Contributions

This research was conceived and designed by SJ, MWP, SH and AM. SJ and EH performed the experiment. The data was analyzed by SJ. MWP and SH contributed the reagents/material/analysis tools. The manuscript was written by SJ, NE and AM.

4.2 Influence of drought and elevated air temperature on pollen characteristics of grasses

Jung, S., Estrella, N., Pfaffl, M. W., Hartmann, S., Ewald, F., & Menzel, A. (2021). Impact of elevated air temperature and drought on pollen characteristics of major agricultural grass species. *PLOS ONE*, *16*(3), e0248759. https://doi.org/10.1371/journal.pone.0261879, IF 3.5 (2021).

Abstract

Grass pollen allergens are known to be one of the major triggers of hay fever with an increasing number of humans affected by pollen associated health impacts. Climate change characterized by increasing air temperature and more frequent drought periods might affect plant development and pollen characteristics. In this study a one-year (2017) field experiment was conducted in Bavaria, Germany, simulating drought by excluding rain and elevated air temperature by installing a heating system to investigate their effects primarily on the allergenic potential of eight selected cultivars of the two grass species timothy and perennial ryegrass. It could be shown for timothy that especially under drought and heat conditions the allergen content is significantly lower accompanied by a decrease in pollen weight and protein content. In perennial ryegrass the response to drought and heat conditions in terms of allergen content, pollen weight, and protein content was more dependent on the respective cultivar probably due to varying requirements for their growth conditions and tolerance to drought and heat. Results support recommendations which cultivars should be grown preferentially. The optimal choice of grass species and respective cultivars under changing climate conditions should be a major key aspect for the public health sector in the future.

Author Contributions

This research was conceived and designed by SJ, MWP, SH and AM. SJ and FE performed the experiment. The data was analyzed by SJ. MWP and SH contributed the reagents/material/analysis tools. The manuscript was written by SJ, NE and AM.

4.3 Influence of land use and cultivation intensity on grass pollen concentration

Jung, S., Yuan, Y., Stange Del Carpio, M., Pawlik, T., Hartmann, S., Estrella, N., Oteros, J., Traidl-Hoffmann, C., Damialis, A., Buters, J., Menzel, A. (2022). Impact of Local Grasslands on Wild Grass Pollen Emission in Bavaria, Germany. *Land*, *11*(2), 306. https://doi.org/10.3390/land11020306, IF 4.0 (2021).

Abstract

Meteorological conditions and the distribution of pollen sources are the two most decisive factors influencing the airborne grass pollen concentration. Especially knowledge about land use types, which potentially emit pollen, and on the relevance of local sources is still limited. In this study, wild grass pollen concentrations from 27 stations in Bavaria, Germany, were linked to potential pollen sources in the respective 30-km radius surrounding. Agricultural grass pollen sources were derived from the database InVeKos providing detailed information on agricultural land use types and their spatial distribution. Non-agricultural grasslands were identified by OpenStreetMap. Further source classification was conducted by using a cultivation intensity indicator and wind direction. We show that the grassland percentage and the pollen concentration specified as Annual Pollen Integral API and Pollen Peak PP were highly variable between the pollen stations. Correlation analyses indicated that the impact of the grassland on the pollen concentrations was larger from the vicinity of the pollen traps until 10 km. With longer distances, the correlation coefficient between the grassland percentage and the pollen indicators steadily declined.

Author Contributions

This research was conceived and designed by SJ, YY, NE and AM. The data was acquired by JO and curated by SJ, YY, TP and SH. The formal analysis was performed by SJ, YY and MSDC and visualized by SJ and YY. The original manuscript was written by SJ and reviewed and edited by all coauthors.

4.4 Use of twig method in climatic chambers for pollen studies

Jung, S., Zhao, F., & Menzel, A. (2021). Establishing the twig method for investigations on pollen characteristics of allergenic tree species. *International Journal of Biometeorology*, *65*(11), 1983–1993. https://doi.org/10.1007/s00484-021-02154-5, IF 3.5 (2021).

Abstract

The twig method in climate chambers has been shown to successfully work as a proxy for outdoor manipulations in various experimental setups. This study was conducted to further establish this method for the investigation of allergenic pollen from tree species (hazel, alder, and birch). Direct comparison under outdoor conditions revealed that the cut twigs compared to donor trees were similar in the timing of flowering and the amount of pollen produced. Cut twigs were able to flower in climate chambers and produced a sufficient amount of pollen for subsequent laboratory analysis. The addition of different plant or tissue fertilizers in the irrigation of the twigs did not have any influence; rather, the regular exchange of water and the usage of fungicide were sufficient for reaching the stage of flowering. In the experimental setup, the twigs were cut in different intervals before the actual flowering and were put under warming conditions in the climate chamber. An impact of warming on the timing of flowering/ pollen characteristics could be seen for the investigated species. Therefore, the twig method is well applicable for experimental settings in pollen research simulating, e.g., accelerated warming under climate change.

Author Contributions

SJ, FZ and AM conceived and designed the study. SJ carried out the experiment and analyzed the data. The original draft was prepared by SJ and reviewed and edited by SJ, FZ and AM.

5 Discussion

5.1 Comparison of pollen characteristics of agricultural relevant grass species / cultivars

Allergen amount is not a linear function of pollen count, largely due to species- or cultivar-specific differences in addition to year, timing, location, and other environmental factors (Aloisi et al. 2019; Maya-Manzano et al. 2022; Buters et al. 2015; Cecchi 2013). Within the scope of this work, particular attention was given to grass pollen due to its generally high allergenic potential (Andersson & Lidholm 2003). The objective was to develop recommendations for the cultivation of grass species and cultivars for agriculture from the point of view of pollen allergenicity. The most important grass species and cultivars used in agriculture in Germany were investigated with regard to their differences in pollen properties, but especially in allergen content. For the determination of pollen characteristics in the laboratory, it was necessary to collect a sufficient amount of pollen. However, problems were caused by insect infestation, moisture and mold growth in the delicate glassine bags in which the pollen was collected on site. To overcome these problems, harvesting of the inflorescences of the grasses took place before flowering and the pollen ripened in a climate chamber. This ensured that sufficient pollen material was available from all grasses to be studied.

The quantification of the group 5 allergen Phl p 5 is comparatively simple (Zimmer et al. 2022; Chapman et al. 2008), as commercial immunodetection (ELISA) test kits are available (Schäppi et al. 1999). Differences in Phl p 5 allergen content were found between individual cultivars from the species Kentucky bluegrass, perennial ryegrass, timothy, cocksfoot, annual / Italian ryegrass, hybrid ryegrass and festulolium of up to 74-fold (timothy cultivar Rubato in comparison to perennial ryegrass cultivar Abervaron), documenting a very high variability (Jung et al. 2018).

In the recorded phenological stages after BBCH (Meier 2001), there were differences of up to 25 days in the respective flowering dates (BBCH 61) of the individual species / cultivars. However, a statistical relationship between allergen content and flowering time could not be found. The values measured in this study for average pollen weight and average protein content were higher than those measured in other similar studies, with an average value for pollen weight of 20.0 ng compared to 11.0 ng (Schäppi et al., 1999) and for average protein content of 12.1% compared to 2.6% (Schäppi et al., 1999) and 8% (Suphioglu et al. 1993), respectively. These differences can be attributed to the fact that the grasses examined in this study were cultivated and not wild grasses and that different standards albumin (Jung et al. 2018) and bovine (Schäppi et al. 1999) were used for protein determination.

The average value of 3 $\mu g.mg^{-1}$ for timothy grass allergen content Phl p 5 measured in this study, which is used as a reference for grass allergy studies, is consistent with concentrations measured in other studies, which ranged from 2.7 to 3.5 $\mu g.mg^{-1}$ (Visez et al. 2021).

Compared to the ryegrass cultivars included in the study, the allergen content of the timothy cultivars studied was significantly higher. At the same time, the timothy cultivars were characterized by high pollen production. Both factors speak against large-scale timothy cultivation purely from the point of view of pollen allergies. Other factors such as forage quality, site-specific species / cultivars mix that ultimately determine the agricultural yield of farmers must be weighed against a reevaluation of species / cultivars from an allergological perspective, which forms an additional challenge for agriculture. Nevertheless, allergenicity considerations should be included in a concept for adapted species / cultivars also in the background of climate change. Compared to timothy, the variation among cultivars in pollen production and allergen content was greater in ryegrass. This may be because five ryegrass cultivars but only two timothy cultivars were studied. The results for pollen characteristics of ryegrass and timothy were confirmed in the independently conducted drought-warm experiment (Jung et al. 2021a).

It was also found that allergen levels increased in the cross-bastard hybrid fescue (ryegrass x meadow fescue cultivar) compared to ryegrass. Due to technical problems caused by early cutting of the grass plots in the following year, results were limited to a single year of measurements of allergen content per species and cultivar. Since the grass species / cultivars studied are perennial, an analysis of allergen content and pollen production in further years would be useful to find out if these change over time.

5.2 Influence of drought and elevated air temperature on pollen characteristics of grasses

The development of plants, and therefore the development of pollen, is determined by meteorological conditions. Increasing periods of drought and heat in the wake of climate change may alter the intensity of the pollen season (Kurganskiy et al. 2021; Zhang & Steiner 2022) and influence pollen characteristics (Reid & Gamble 2009). In the framework of this work, the effects of drought and warming before and during flowering on the timing of flowering and pollen characteristics in different cultivars of timothy and ryegrass species were studied. In the 2017 manipulation experiment in Dürnast, transparent mobile rainout shelter simulated drought by omitting any precipitation during the investigation period and was able to reduce soil moisture by 28%. The increase of the air temperature near the ground could be simulated by the special design of the heating system in the period from May 19 to June 23 with an increase of the air temperature on average by 0.87 °C.

The timing of flowering in the elevated air temperature treatments, for both ryegrass and timothy species occurred on average about one day earlier per 1 °C which is consistent with the study of Menzel et al. (2021). In comparison, flowering was latest in the drought treatment, which can be explained by a reduction in plant vigor and a change in pollen development (Cui et al. 2017; Castillioni et al. 2022; Craine et al. 2012).

Overall, there was a strong influence of elevated air temperature and drought on pollen characteristics, especially on allergen content, but the effect depended strongly on the species and the particular cultivar. In timothy, drought treatments resulted in reductions in pollen weight, protein content, and allergen content. A rather low adaptability of timothy to changing environmental conditions such as elevated air temperatures has already been shown in another study (Hanna et al. 2018). In ryegrass, the effect on pollen characteristics was much more variable among cultivars. However, a tendency toward higher allergen levels was observed for most ryegrass cultivars. For the other pollen parameters pollen weight, protein content, no consistent effect was found for ryegrass in contrast to timothy.

The loss of plant vigor in the drought-treated plots was readily apparent from the drop in measured green value between May 30 and June 12, 2017. This reduction in plant vigor may be the reason for the reduction in pollen weight, protein content, and allergen content of timothy, which may be part of the trade-off within plants that has already been observed in other plant species (Berger et al. 2017). Compared to timothy, ryegrass flowering started an average of 7 days earlier, resulting in less loss in plant vigor in ryegrass at flowering than in timothy. If the loss of plant vigor had been more advanced, the effect on ryegrass might have been similar to that on timothy with a drop in allergenicity.

Another explanation for the differential response of ryegrass cultivars compared to timothy cultivars to drought/elevated air temperatures could be the adaptation to specific environmental conditions, suggesting that the effects of the additional treatments were partially masked by intercultivar variability. Individual species / cultivars may have different tolerance levels to drought and elevated air temperature (Craine et al. 2013). These tolerance levels can determine whether allergen levels increase, as seen in most ryegrass cultivars, or decrease, as seen in most timothy cultivars.

From the perspective of climate change associated with the identified response to drought and an increase in air temperature, timothy should be preferred to ryegrass in cultivation because of the resulting reduction in allergen content. Arguing against this is the fact that the allergen content of timothy grass is naturally five times that of ryegrass, and timothy also produces 30-times more pollen. Elevated air temperature and drought had no significant effect on pollen production of the grass species studied, there were only differences between individual cultivars. In other studies, CO_2 fertilization has been identified as a factor influencing pollen concentration so that plants undergo increased photosynthesis and subsequently grow better and produce more pollen (D'Amato et al. 2020; Cecchi et al. 2018; Zhang & Steiner 2022). It is possible that there are species- and cultivar-specific differences in the response to CO_2 fertilization and that not all plants necessarily produce more pollen. Therefore, various factors must be considered in order to keep the pollen load for allergy sufferers as low as possible. The cutting dates must also be included here, since timothy, for example, blooms later than ryegrass and the possibility is higher that it will already be cut before flowering. Overall, the effect of drought / elevated air temperature on pollen characteristics was found to depend strongly on the species and the particular cultivars studied.

The elevated air temperature of 0.87℃ added to the treatments corresponded to a moderate change in environmental conditions due to climate change, with the temperature increase roughly corresponding to the Representative Concentration Pathways (RCP) 2.6 scenario (Global average temperature increase below 2℃ by 2100). For the future, even the RCP 4.5 or RCP 8.5 scenario should be considered.

Drought added during treatments provided soil moisture which was on average 28% lower than the control. In Germany, droughts are becoming more frequent even in spring, for example in April 2020 the usable field capacity was reduced to 68% on average (Imbery et al. 2021). It would be necessary to find out in further experiments what would happen if the changed environmental influences were stronger and persisted over an even longer period of time. In the present study, the lowest soil moisture was <0.04 m^3 m^{-3}. If, in further experiments, the simulated drought was even more pronounced, reaching the wilting point (pF < 4.2) and taking into account the soil type, individual species might not be able to flower or even form inflorescences.

In the long term, changing environmental conditions may alter the species composition and thus the amount of pollen released and its allergenic potential (Weger et al. 2013). Despite the rather moderate change in environmental conditions in this study, these were already sufficient to have a significant impact on pollen characteristics, including allergenicity. Given the different responses to the treatments added depending on the species/cultivar, this information should be included in future cultivar recommendations. New strategies are being sought for agriculture, including the breeding of cultivars adapted to the changed environmental conditions (Lin 2011; Rognli et al. 2021). Since many other parameters such as yield and quality are of primary importance for agricultural production, seed mixtures adapted to allergen content would be feasible, at least on extensively used land. This study can be part of the considerations to develop strategies to reduce the allergy risk for pollen allergy sufferers, while weighing other aspects such as biodiversity conservation, peatland rewetting with an integration of other grass communities or carbon sequestration.

5.3 Influence of land use and cultivation intensity on grass pollen concentration

As explained above, pollen production and thus the amount of pollen emitted is highly dependent on the species/cultivar and is also influenced by the prevailing environmental conditions. The influence of land use on the wild gras pollen concentration and in particular on API (Galán et al. 2017) and PP (Oteros et al. 2015) was studied as another important factor. Both the influence of distance and cultivation intensity were evaluated.

The comparison of wild grass pollen concentrations expressed as API and PP and the potential pollen source areas in a correlation analysis revealed that much of the influence is limited to the surrounding 10 km. A study using HYSPLIT models to determine which source areas might contribute to pollen concentrations also found that the largest area of influence in rural areas was within a 2-10 km radius (Frisk et al. 2022). The two pollen concentration indices studied, API and PP, showed high correlation ($p<0.0001$, adj. $R2$ 82.6%) except for two alpine stations. Grassland categories within the InVeKos database differ greatly in their cultivation intensity, resulting in large differences in the amount of pollen released. When considering the different categories, the respective cultivation intensity should therefore also be taken into account. At least 33 different categories of grasslands exist in the database. The majority of grasslands (~90%) belong to agricultural holdings as shown in the analysis of InVeKos and OpenStreetMap. Among the grassland categories, meadows and mowing pastures, both of which are subject to high cultivation intensity for the most part and thus have greatly reduced pollen emission, occupy the largest share with a total of 71% of the area. In comparison, only a relatively small portion is extensively farmed, which is mostly found in grassland categories with low area percentages. However, it can be assumed that areas with extensive management such as field margins or Natura 2000 protected areas are of great importance for pollen concentrations despite their small share. In cultivation intensity, a high range was observed, ranging from non / extensively managed to very intensive management with up to seven cuts per year. In light of the fact that many of the grass species are not able to flower and release pollen again after the first cut, this cut is most meaningful for pollen concentrations. It has already been demonstrated in another study that earlier cut grasslands have a lower amount of emitted pollen (Jetschni & Jochner-Oette 2021).

It can be assumed that the Pearson correlation coefficients between grass pollen concentration and land use which were 0.36 for API and 0.33 for PP at the maximum would be significantly higher when considering individual species. However, the measured grass pollen concentrations are composed of a variety of different grass species. Species composition can vary greatly from station to station due to local or regional differences in habitat requirements, including elevation or soil-climate zone (García-Mozo et al. 2016). Using the technique of high-throughput sequencing, it has been shown for the UK that pollen species composition varies with latitude and longitude, in addition to variability during the pollen season (Brennan et al. 2019). In addition to pollen production, species-specific flowering dates also vary, which means that the proportion of early- or late-flowering grass species in the species compositions can vary. In terms of cultivation intensity, this means that even under high cultivation intensity, more grasses may come to flower in species compositions with a high proportion of early flowering species. In the present correlation coefficients, it may also have played a role that on micro scale level very small grass plots, as they are found in large numbers everywhere, could mostly not be recorded. Especially these small areas are mostly not cultivated and can significantly influence the pollen concentration due to their large number.

It would actually have been expected that the innermost rings would have the greatest influence on pollen concentration, since they reflect the local vegetation in the immediate vicinity of the pollen trap. Instead, the distance of 2.5 km had the greatest influence on the pollen concentration for API and PP. This can be explained by the fact that, due to the smaller ring sizes in the inner rings, the potential pollen sources are distributed much more variably here than in the rings located further out.

The positioning of the pollen trap can be mentioned as another influencing factor. Following the international standards, the pollen traps are installed 15 meters above the ground (Hjort et al. 2016), which implies that suitable buildings must be found for this purpose. Since these buildings are mostly operated by public institutions, they are usually located in more populated areas (Rojo et al. 2019a) where it can be assumed that the potential source of pollen emission is rather low.

Another question was to evaluate the extent to which cultivation intensity and wind direction influence the correlation between grassland percentages and pollen concentrations. As a result, neither cultivation intensity nor wind direction had a strong influence. For cultivation intensity, this can possibly be explained by the fact that the area shares for intensive, moderate, and extensive are generally different in size. Regarding the wind direction, it should be noted that some of the measuring stations were located at a greater distance from the pollen measuring stations. In order to be able to investigate the influence of the wind direction on the pollen concentration more precisely, the measurement of the wind direction in the immediate vicinity of the pollen trap would be absolutely recommended. Instead of dividing the wind direction into four quarters (NE, SE, SW, and NW), it would be possible to apply a division into 360 sections.

A sensitivity analysis using OpenStreetMap grassland weighted by station-specific annual pollen concentrations was used to determine the extent to which correlation coefficients change. Compared to the conventional weighting with cultivation intensity and wind, there were improvements in the correlation coefficients in the middle to far distances from the stations in

the sensitivity analysis. Based on the rather small improvements at closer distances, it can be seen that the applied weighting with cultivation intensity reflects the pollen concentration in the closer surroundings well while this is not the case at longer distances.
This study considered the current state of land use, while changes in land use could significantly affect pollen concentration. These changes are site-specific and can include for Europe both expansion or reduction of agricultural grassland and changes in the intensity of land management, among others (van Vliet et al. 2015), though for Germany it was found that the intensity of land use has remained unchanged at a high level in recent decades (Kuemmerle et al. 2016). Nevertheless, the influence of land use change on pollen concentration should be further investigated.

5.4 Use of twig method in climatic chambers for pollen studies

Pollen studies that require the collection of pollen during flowering outdoors in the field are often faced with many challenges, including the influence of weather conditions. The present study successfully demonstrated that the twig method in climatic chambers is not only suitable for phenological studies (Basler & Körner 2012; Primack et al. 2015; Polgar et al. 2014; Menzel et al. 2020a) such as determining the influence of winter (cooling) temperatures (Laube et al. 2014a) and bud development at elevated humidity (Laube et al. 2014b), but also for pollen studies. The set temperature day-night cycle of 15 (20 °C air temperature) and 9 h in the climatic chamber allowed the flowering of twigs with inflorescences of the shrub species hazel and tree species alder and birch. Sufficient pollen was collected for subsequent laboratory testing in the glassine bags previously placed over the inflorescences.
It was also investigated whether the cutting of the twigs necessary for ripening in the climatic chamber had an influence on the parameters studied. The comparison made for this purpose between cut and uncut twigs under outdoor conditions showed minor, no significant differences in the parameter’s catkin length, pollen weight per catkin and protein content. For the two other studied parameters, namely allergen content per pollen grain and pollen weight, there were generally larger deviations, but this can be attributed to the comparatively small number of samples. Flowering occurred on the same day in both cut and uncut twigs. Overall, it could be concluded that pollen produced from cut twigs are suitable for pollen studies.

Also studied was the use of various additives for fertilization and an agent against fungal growth. Unlike other studies (AL-Kahtani & Ahmed 2012; Maksoud 2000), no significant difference was found in this study between pure water, plant fertilizer and tissue fertilizer in the twigs in the climate chamber or windowsill for plant vitality or other parameters. Accordingly, it is sufficient to change the water once a week and to counteract possible mold growth (Criado et al. 2005) by adding a remedy.
It was also investigated whether twig experiments in climate chambers can be used to perform various climate manipulations that are representative of altered outdoor climate conditions. The twigs cut for this purpose at regular intervals up to four weeks before the actual flowering and stored in the climate chamber, reached flowering and pollen was released. Flowering occurred much earlier for the twigs in the climate chamber (average temperature 16.8 °C) than for the selected twigs in the field (average temperature 3.7 °C). After harvesting the twigs flowering occurred within 5-10 days in the climate chamber. Among the species studied, flowering occurred up to 69 days earlier in the climate chamber for hazel, 35 days for alder, and 15 days for birch, which could be seen also in another study (Wang et al. 2020). An earlier flowering date in the climate chamber / windowsill compared to outdoor conditions was reflected in lower pollen production per catkin, catkin length and protein content for the parameters studied. In birch, the same relationship was additionally found in an indoor-only comparison between the harvest dates of March 12 and April 9 of the twigs, with the later harvested twigs also showing higher values for the parameters studied, which has already been demonstrated in another study for allergen content (Buters et al. 2010). By reaching flowering in the climate chamber at different times before flowering under ambient conditions and producing sufficient pollen for the laboratory analysis (>10 mg), climate chamber experiments on twigs are suitable for climate manipulations. This would be applicable as a proxy for various field manipulations for pollen research such as different climatic scenarios for temperature, humidity, ozone, and CO_2 (Primack et al. 2015; Vitasse & Basler 2014). These types of experiments, for example, can make an important contribution to the study of the influence of winter temperatures on spring phenology.

6 Outlook

In the context of the present work, we have had to limit the study to selected grass species. In order to be able to give even more comprehensive recommendations for possible seed mixtures in agriculture, it would be useful to investigate further cultivars of ryegrass due to their high number and large-scale cultivation. In the case of timothy, it should be investigated whether other cultivars, in addition to those already studied, also exhibit such a high allergenic potential. Individual species not included so far, such as meadow foxtail, which are also widespread, should also be investigated. In addition, grass species that grow extensively on uncultivated land should be studied more closely. In the present study, only pollen from the first flowering was examined. Further studies should investigate what proportion of the total pollen concentration is accounted for by the second and possibly third flowering, whereby it would first be necessary to check whether the respective species and cultivars release pollen at all a second time. Furthermore, it should be analyzed whether the pollen characteristics change at the second and third flowering.

Changes in environmental conditions due to climate change, associated with an increasing number of extreme events, will affect plant growth and pollen characteristics. The effects studied in this work focused on drought and elevated air temperatures, and their effects on pollen characteristics of annual grass plants. Pollen characteristics can change during the life cycle of plants, so it would be useful to study the impact of drought and elevated air temperatures over several consecutive years. In addition, it would be useful to study grasses of different provenances, as grasses differ genetically or phenotypically and may respond differently to added stress (Knapp et al. 2001). As a counterpart to drought, the influence of flooding as an extreme event and its impact on pollen characteristics of different species and cultivars should be studied.

The influence of land use on pollen concentration has been investigated macroscopically for the whole of Bavaria in this work. Main influence of land use was narrowed down to the surrounding 10 km. More research would be needed to determine which land use types emit the most pollen. Research would still need to be conducted to determine the influence of land use at much smaller scales when pollen stations are placed within a few hundred meters of each other and at a few meters above the ground.

Finally, with the establishment of the twig method in climate chambers, the prerequisite has now been created for future pollen studies to be carried out under a wide range of defined environmental conditions.

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(2) Impact of elevated air temperature and drought on pollen characteristics of major agricultural grass species. https://doi.org/10.1371/journal.pone.0261879

(3) Impact of Local Grasslands on Wild Grass Pollen Emission in Bavaria. https://doi.org/10.3390/land11020306

(4) Establishing the twig method for investigations on pollen characteristics of allergenic tree species. https://doi.org/10.1007/s00484-021-02154-5

PLOS ONE

RESEARCH ARTICLE

Grass pollen production and group V allergen content of agriculturally relevant species and cultivars

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Abstract

Grass pollen is the main cause of hay fever and allergic asthma in warm temperate climates during summer. The aim of this study was to determine the content of group 5 major allergens in pollen grains of agriculturally important grass species/cultivars. For each cultivar flowering dates and pollen production of cut anthers were observed in the field and in a climate chamber, respectively. An ELISA was used to quantify the group 5 allergens (Phl p5) in pollen extracts which were gained from the grass species Kentucky bluegrass, perennial rye grass, timothy, cocksfoot, annual / Italian rye grass, hybrid rye grass and festulolium. The group 5 allergen content of species varied between 0.01 ng (Kentucky bluegrass) and 0.06 ng (timothy) per pollen grain. On cultivar level the pollen allergenic content differed up to 74-times within the selected grass species. Results from this study might be helpful for the reduction of allergen exposure coming from agriculture grass production e.g. by an adapted grass selection or by the cultivation of grasses with low allergenic content in plant breeding.

Introduction

Grass pollen is, besides birch pollen, one of the most frequent reasons for plant related allergenic reactions worldwide [1]. People who are sensitive or allergic to pollen develop symptoms such as hay fever, allergic rhinitis or even allergic asthma. At present 13 different groups of allergens related to grass pollen are known which induce IgE responses [2], of which 11 groups can be found in *Pooideae* pollen, a subfamily of the *Gramineae* or *Poaceae* family [3]. Among these allergens, group 1 (glycoproteins, molecular mass of 32–35 kDa) and group 5 allergens (glycoproteins molecular mass 28–32 kDa) are the major allergens, since they induce allergenic reactions in patients at high rates (65–85% of patients allergic to grass pollen are sensitive to group 5 and 90–95% to group 1 allergens) [3–5]. Grass pollen (~35–65 μm in diameter) itself is not tiny enough to enter lower airways and thus to directly cause respiratory allergies. Rather, when pollen is dissolved its allergenic content is released in form of small granules

(0.6–2.5 μm). These granules can bind to smaller particles, e.g. fine dust, and thus are small enough to cause allergenic reactions, also in the distal parts of the lungs [6].

Worldwide there are 9,000 different grass species (*Poaceae*) [2], nevertheless in German agriculture only about 20 of them achieve higher coverage in the area. But in fact one species may comprise lots of varieties. For example, about 300 different cultivars of perennial rye grass (*Lolium perenne* L.) are listed in Germany. Cultivars have been developed by plant breeders for specific purposes, such as maximizing agricultural yield under certain site conditions or ornamental aims. Thus, pollen allergenicity of different cultivars has been—to our knowledge completely—neglected due to its limited relevance in agriculture and horticulture. Limited data only exists on the species level (e.g. [7]).

In general, pollen concentrations in the air are highly dependent on the timing and duration of flowering as well as short- and long-range atmospheric transport and weather conditions. For grass, agricultural management is another impact factor, since hay cutting or in more recent time's silage cutting might take place before most grass species reach the flowering phase, resulting in a considerable smaller pollen release. Warmer late winter / early spring conditions due to anthropogenic induced climate change advance the spring phenology of many plant species in the temperate and boreal zone [8], therefore also grass species have shown a trend towards earlier flowering in the recent decades [9]. For Germany, most interestingly, a divergence between flowering and hay cutting dates has been revealed [10]. More specifically, flowering dates tended to be earlier whereas hay cutting dates did not advance during the last decades. This phenomenon can be explained by management decisions among others historically fixed hay cutting dates or subsidies [11] in Agri-Environmental schemes for extensification (later first silage cutting leading to reduced number of cuts per year or land set-aside) to e.g. preserve biodiversity [12]. If farmers' annual activities are not tracking the speed of climate change in their grassland management [13], this divergence would allow at the end more grass species to come into flowering and consequently more allergenic pollen to be released into the atmosphere. On the other hand, due to the fact that hay production was largely replaced by silage production and thus there are an increased number of cuts per year in the past decades, the first silage cut should be antedated. The number of cuts has a significant influence on the annual agricultural income [14]. Additionally, from the agricultural perspective it is very important that the silage cut should take place when ear/panicle emergence of the dominant species in the sward (pointed foxtail *Alopecurus pratensis*) occurs as the fodder quality immediately decreases after flowering [15,16]. Potential adaptations in agriculture management towards climate change from farmers side depend on their awareness [17]. Anyhow, strategies for a sustainable intensification in management are already discussed [18].

Since the number of persons allergic to grass pollen is high and still increasing, more effort should be put into the investigation of the allergen content in species and cultivars in order to provide improved allergen-specific recommendations in the selection and cultivation of cultivars, not only in agriculture, but especially in landscape building, gardening, and urban landscaping as these cultivation forms are exclusively driven by seedlings. Studies on mean allergen exposure mostly rely on daily pollen concentrations/counts derived from volumetric pollen samplers. At present an optical identification and distinction of pollen from different grass species is not possible [19], although most grass species do not cause allergies at all. Therefore grass pollen counts only to a certain degree mirror the related allergenic potential. Furthermore, the allergenic potential strongly depends on weather conditions, season of the year and geographical location making it difficult to predict allergen exposure only by pollen concentration [20].

Therefore, it is important to know to which degree pollen of the selected grass cultivars vary in their amount and allergen content, and whether allergen content and pollen production are related to flowering dates of the cultivars.

To address this question we chose 15 different grass cultivars due to the allergenicity of their pollen [21], their prevalence in seed mixtures, different heading date, ploidy and genetic background. We collected grass shortly before flowering on a grass cultivar trial field in 2016 and analyzed the amount of pollen produced, and group 5 allergenic content of the pollen.

The amount of major group 5 allergens on species/cultivar level was determined by molecular techniques [22], and pollen productivity was analyzed by pollen count. Phenology and particular flowering dates were recorded according to the BBCH code [23]. Results show that there are large differences in allergenic content between the selected cultivars. They may help to optimize the choice of grass species, to revise agricultural and landscape management practices and/or support plant breeders in their efforts to breed cultivars with down-regulated allergens [24].

Material and methods

Investigated grass species and cultivars

The following seven grass species were analyzed for their allergenic content: Kentucky bluegrass (*Poa pratensis* L.), perennial rye grass (*Lolium perenne* L.), timothy (*Phleum pratense* L.), orchard grass (*Dactylis glomerata* L.), annual / Italian rye grass (*Lolium multiflorum* Lam.), hybrid rye grass (*Lolium x hybridum)*, and festulolium (*Festuca spec. x Lolium spec.*). In total 15 cultivars were chosen for this study, one annual and one hybrid rye grass, two cultivars each of Kentucky bluegrass, orchard grass, timothy and hybrid fescue as well as five cultivars of perennial rye grass (see Table 1 for a complete list of the cultivars). All grass cultivars used in this study passed Distinctness, Uniformity and Stability (DUS) procedures, thus it is guaranteed, that varieties are authentic variants from the respective species. The seeds of the cultivars planted by Bavarian State Research Center for Agriculture (LfL) have the quality level of seeds used in Value or Cultivation and Use (VCU)-trials. So seeds of high defined genetic standard were ordered directly from the breeder or the responsible maintainer of the respective variety. The cultivars selected vary mainly in their flowering time.

Experimental site

The experimental field site was located at 450 m a.s.l. in Pulling (southern part of Germany, close to Munich / Freising, 48.3712˚N, 11.7181˚E) and is operated by the LfL. This trail field site exists since 2014 and consists of various grass species and cultivars which are grown for mainly educational purposes. Each cultivar is planted on a 1.5 m x 7 m subplot.

Phenological, meteorological and airborne pollen data

From beginning of May till end of June 2016 vegetative and reproductive phenological stages of all cultivars (except for festulolium and annual rye grass cultivars, which were included later in the study) were recorded with the expanded BBCH code on a weekly basis following the description of Meier [23]. Observations included the macro stage 4 (booting), 5 (inflorescence emergence, heading), 6 (flowering, anthesis), 7 (development of fruit), 8 (ripening) to macro stage 9 (senescence). The mean weekly (micro-) stage was derived by averaging the (micro-) stages recorded for each of the subplots. Linear interpolation was used to receive the exact flowering dates (BBCH 61).

Table 1. Allergen content of 15 allergenic grass cultivars. Soluble group 5 allergens in solution; mass per pollen grain, soluble protein and group 5 allergens per pollen grain; percentage of soluble protein and group 5 allergens per total grain mass and percentage of soluble group 5 allergens per total soluble protein in pollen extracts; NA = not available (too little of pollen).

Grass species	Cultivar	Soluble group 5 allergen content (μg/ml)	Mass per pollen grain (ng/grain)	Soluble protein per pollen grain (ng/grain)	Soluble group 5 allergen per pollen grain (ng/grain)	Soluble protein per total grain mass (%)	Soluble group 5 allergen per total grain mass (%)	Soluble group 5 allergen per total soluble protein (%)
Kentucky bluegrass	WR Lato	123.42	7.49	0.86	0.009	11.43	0.12	1.08
Kentucky bluegrass	WR Liblue	113.24	12.27	1.52	0.014	12.36	0.11	0.92
Average Kentucky bluegrass		**118.33**	**9.88**	**1.19**	**0.012**	**11.89**	**0.12**	**1.00**
Festulolium	FL Lesana	163.65	19.34	2.75	0.032	14.22	0.16	1.15
Festulolium	FL Paulita	122.51	NA	NA	NA	NA	NA	NA
Average Festulolium		**143.08**						
Hybrid rye grass	BW Pirol	113.52	NA	NA	NA	NA	NA	NA
Italian rye grass	WW Hera	114.86	41.67	5.85	0.048	14.05	0.11	0.82
Cocksfoot	KL Diceros	186.28	16.67	2.11	0.031	12.64	0.19	1.47
Cocksfoot	KL Musketier	89.44	23.95	3.36	0.021	14.04	0.09	0.64
Average Cocksfoot		**137.86**	**20.31**	**2.73**	**0.026**	**13.34**	**0.14**	**1.06**
Perennial rye grass	WD Aberavon	36.31	12.50	1.51	0.005	12.08	0.04	0.30
Perennial rye grass	WD Barata	99.38	12.50	1.47	0.012	11.76	0.10	0.85
Perennial rye grass	WD Borsato	129.79	21.76	2.23	0.028	10.26	0.13	1.27
Perennial rye grass	WD Ivana	96.59	26.53	2.12	0.026	8.00	0.10	1.21
Perennial rye grass	WD Matenga	90.70	27.89	3.21	0.025	11.51	0.09	0.79
Average Perennial rye grasses		**90.55**	**20.24**	**2.11**	**0.019**	**10.72**	**0.09**	**0.88**
Timothy	WL Barpenta	324.39	16.39	2.01	0.053	12.27	0.32	2.64
Timothy	WL Rubato	265.70	23.67	3.06	0.063	12.93	0.27	2.06
Average Timothy		**295.04**	**20.03**	**2.54**	**0.058**	**12.60**	**0.30**	**2.35**
Overall Average		**137.98**	**20.20**	**2.47**	**0.028**	**12.12**	**0.14**	**1.17**

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Airborne pollen concentration was measured during the vegetation period of 2016 using a 7 Day Recording Volumetric Spore Sampler (Burkard Scientific Ltd, Uxbridge, UK) which was installed in 15m height above ground at the forest faculty building of the Technical University of Munich in Freising, Germany (48.3999˚N, 11.7180˚E) and was there attached to the meteorological platform at the east side of the building. The air flow was set to 10 L/min. Pollen grains were trapped on an adhesive tape which was fixed on a drum and driven by a clockwork motor. After recording, the sampling tape was cut into half day sections which were than preserved on microscope slides. Grass pollen were identified and counted by light microscopy following the requirements for pollen monitoring [25].

Meteorological data (air temperature, air humidity and precipitation) in hourly resolution were obtained from a nearby climate station (Weihenstephan-Dürnast, location 48.4029˚N; 11.7305˚E, distance to trial field site 3.5 km and distance to meteorological platform 1.0 km) of the German Meteorological Service (DWD) and used for characterizing the meteorological growing conditions.

Collection of grass pollen and pollen count per culm

When first flowering (BBCH code 61) was observed for a cultivar, bunches of 50–60 individual plants were harvested randomly from the plot and inflorescences were covered with pergamin bags. Pollen collection directly on the field turned out to be impossible due to adverse environmental conditions (rain, herbivore insects). After attaining the full flowering stage (BBCH code 65) under fixed conditions in a climate chamber (day/night cycle: 14 hours day at 23˚C, 10 hours night at 15˚C; air humidity 45%), pollen was extracted from the pergamin bags by shaking and subsequent removal of anthers and culms. Additionally the number of culms per bag was counted and the total pollen weight was determined by an electronic balance (Mettler Toledo, model XS204DR). This method for pollen collection and isolation similar to [26] was consistently applied for all samples. However we cannot exclude that single pollen still adhered to the bags or were not released by the anthers at that time.

Extraction and determination of protein content

100 mg pollen grains were mechanically decomposed by shaking pollen continuously at room temperature for three hours in PBS (phosphate buffered saline) [27,28]. Afterwards the dispersion was centrifuged at 13.600 rpm for 5 min. Analysis of total soluble protein content was conducted with a classical BCA test [27,28]. BCA solution and copper sulfate were obtained from Sigma (B9643; C2284). For the standard curve Albumin from Serva (11930) was used and its range was 25–1000 μg/ml concentration.

Grass pollen weight and allergen quantification

5 mg pollen grains of each grass cultivar sample were weighed, dissolved in 250 μl PBS and then the amount of pollen were counted (n = 4) by an automated cell counter (TC-10, Bio-Rad Laboratories GmbH, München, DEU) [28]. Results from these four counts were then averaged. Group 5 allergen quantification was carried out by a commercial available sandwich ELISA (Allergopharma GmbH, Reinbeck/Hamburg, Germany) [7,22,29]. Within this test the timothy (*Phleum pretense*) group 5 allergen Phl p5 served as reference [30] and the monoclonal antibodies MoAb 1D11 and MoAb B01 (Allergopharma GmbH) were used to detect the available epitopes present on the grass pollen allergens Phl p5a and Phl p5b. The biotinylated second antibody B01 together with a chromogen generated spectrophotometrically detectable signals visualizing the epitope-antibody binding. Based on the standard material a standard curve from 1 to 1000 ng/ml concentration was created. The allergen quantification of all group 5 allergen by ELISA allowed a sensitivity of 1ng/ml and showed a precision of ±10% [31]. The antibodies MoAb 1D11 and MoAb B01 can be used for the quantification of group 5 allergen content in soluble extracts from all species of *Poaceae* as group V allergens are homologous proteins in grass pollen [32,33].

Statistics

The data were analyzed with R [34] using the packages ggplot2, latticeExtra, tidyr and ggpubr. Data were tested for normal distribution using the Shapiro test. Correlation analysis for non-parametric data was calculated by Spearman's Rank Correlation. P values smaller than 0.05 were considered as statistically significant.

Results

Airborne pollen counts and weather in spring 2016

In the sampling period (03.03. - 15.10.2016), an average daily concentration of 21 grass pollen m^{-3} and a total amount of 4,800 pollen m^{-3} for the whole vegetation period were measured.

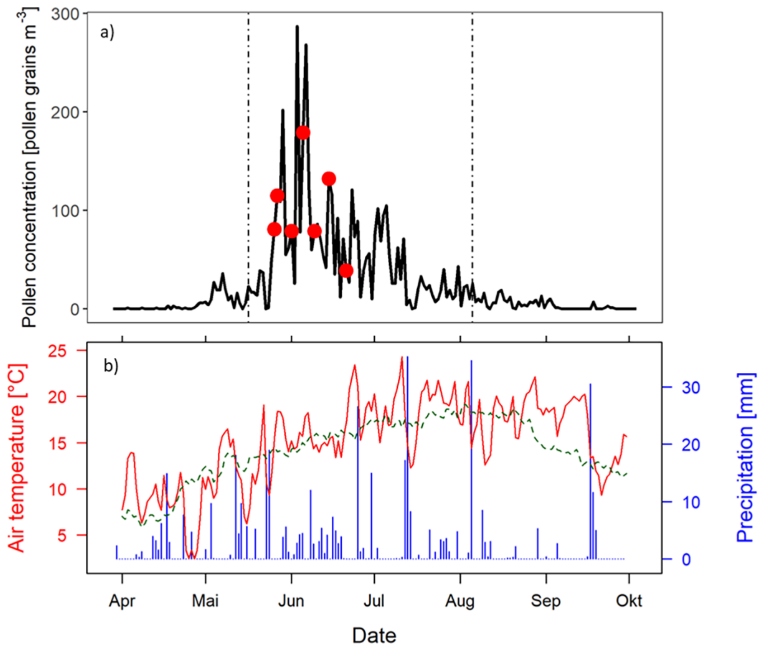

Fig 1. a) Pollen concentration (pollen grains m^{-3}). Dashed lines indicate thresholds when 5 and 95% of grass pollen are released. Red points represent start of flowering for the investigated cultivars b) Air temperature (˚C) and precipitation (mm) from March 3rd to October 15th 2016. Green dashed line shows averaged air temperature between 1987 and 2016.

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Start and end of grass pollen season (5% and 95% of year's total amount reached) were on 18.05.2016 and 04.08.2016. Highest grass pollen concentrations with up to 268 pollen m^{-3} per day (04.06.2017) were reached between end of May and mid of June (see Fig 1). Mean air temperature in the sampling period was 13.3˚C, and precipitation was around 500 mm in total. In comparison to the average climate conditions from 1987 to 2016, air temperature in 2016 was around 0.5˚C higher and total precipitation was nearly 60 mm lower. For April, May and June average monthly air temperatures of 8.4˚C, 12.9˚C and 16.6˚C were measured. The precipitation was 49 mm in April in comparison to Mai and June (100 mm and 105 mm) (Fig 1).

Phenology

Among the selected cultivars, Ivana (perennial rye grass cultivar), Liblue and Lato (Kentucky bluegrass cultivars) and Musketier (orchard grass cultivar) were the first to start flowering (BBCH code 61) on May 27 and 28 (day of the year (DOY) 148 and 149) (Fig 2). The first flowering for Diceros (cocksfoot cultivar) was determined on DOY 154. For Matenga, Barata and Aberavon (perennial rye grass cultivars) flowering was observed between DOY 158 and 162 (middle to late flowering cultivars). Borsato (perennial rye grass cultivar) and Rubato (timothy cultivar) started flowering at DOY 162 and 167 (late). Barpenta (timothy cultivar) flowered latest on June 21 (DOY 173). Independent from cultivar, Kentucky bluegrass and cocksfoot flowered early and timothy was a late flowering species whereas within perennial rye grass there was a large range of flowering dates of the different cultivars. Since we only have data from one season and one site, thorough statistical analyses are not possible. The first peak in atmospheric grass pollen concentrations matched with the start of flowering of the earliest cultivars, whereas first flowering of the latest cultivars was within the smaller peaks towards the end of the grass pollen season (see Fig 1).

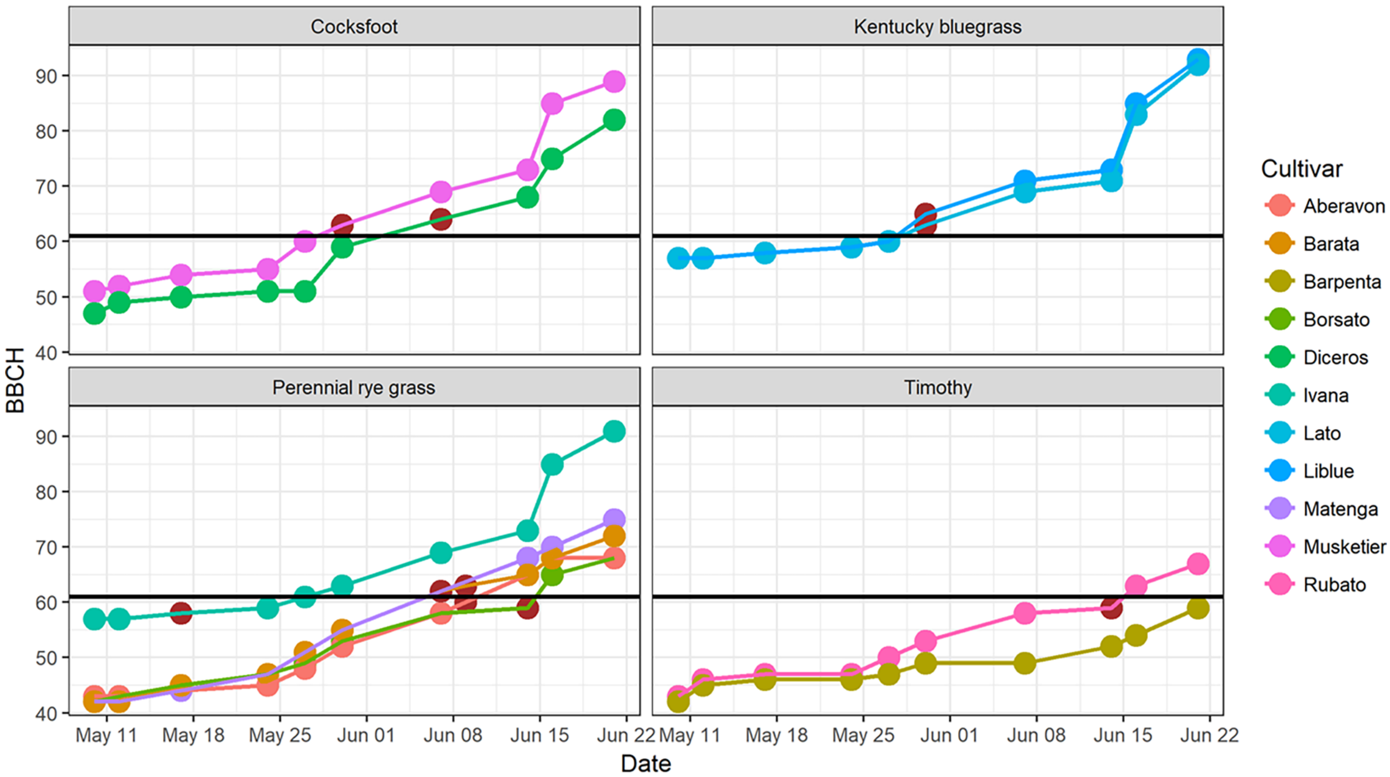

Fig 2. Phenological development of different grass species and cultivars in spring 2016. BBCH indicates the (micro-) stages according to BBCH code [23] between May 10th and June 21st 2016. Black line indicates start of flowering (BBCH 61). Dark brown points represent the date of harvesting on the field for analysis of allergen content.

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Harvesting of plant material for pollen collection in the climate chamber took place shortly after first flowering (BBCH 61) was observed for the early flowering species Kentucky bluegrass (2 cultivars) and cocksfoot (2) as well as for 2 out of 5 cultivars of perennial rye grass. In contrast, for the late flowering species timothy (2 cultivars) as well as for 2 late flowering cultivars of perennial rye grass the harvesting was done shortly before first flowering. Only Ivana (perennial rye grass) was harvested considerably earlier than the first flowering date.

Pollen allergen content

For the investigated cultivars the mean observed pollen grain weight was 20.2 ng (Tab. 1) whereas the highest mass (41.7 ng $grain^{-1}$) was reached by Hera (Italian rye grass cultivar) and the lowest mass (7.5 ng $grain^{-1}$) was found for Lato (Kentucky bluegrass cultivar). These values are matching with total protein content, for which Hera had the highest value (5.9 ng $grain^{-1}$) and Lato again the lowest one (0.9 ng $grain^{-1}$), respectively. In average, pollen extracts had a total protein content of 11.64 μg / ml which is in good agreement to other studies [27]. The mean total protein amount per pollen grain was 2.5 ng.

In the extracts from different cultivars there was on average 138 μg/ml group 5 allergen concentration, ranging—on the species level—from 90 μg/ml (perennial rye grass) to 295 μg/ml (timothy). The lowest allergen content among all cultivars was found in Abervaron (perennial rye grass cultivar) with 36 μg/ml and the highest in Barpenta (timothy cultivar) with 324 μg/ml (Table 1).

On the pollen grain level an average allergen content of 0.03 ng was observed. In relation to grain mass, the group 5 allergen proportion was 0.14%, ranging—on the species level—from 0.09% (perennial rye grass cultivar) to 0.30% (timothy cultivar). On cultivar level the smallest proportion was found in Aberavon (0.04%, perennial rye grass cultivar) and highest proportion was encountered in Barpenta (0.32%, timothy cultivar). Compared to other cultivars, Barpenta was growing in clusters poorly covering ground, and flowering was observed six days after the other cultivars (Fig 2). Allergen proportions correlated reasonably well with flowering dates of cultivars ($r = 0.46$, $n = 11$, $p = 0.15$, not significant), indicating for the later flowering cultivars higher allergen proportions.

On average 5,400 ng group 5 allergen was observed per culm whereby the highest amount was reached by Rubato (14,200 ng, timothy cultivar) and the lowest in Aberavon (192 ng, perennial rye grass cultivar). Thus, Rubato has a 74-times higher group 5 allergen content at culm level in comparison to Aberavon.

Discussion

The group 5 allergen (Phl p5) is one of the largest triggers of hay fever and allergic asthma. Phl p5 is a protein with two different isoforms and high variability of IgE epitopes explaining its high allergenic potential [35]. It can be found exclusively in the grass Pooideae subfamily [36,37]. The determination of allergen content focused on the group 5 as it is well detectable in various grass species using the ELISA technique [7] whereas allergen quantification in other allergen groups is known to be not accurate (group 1 allergens) or allergen potential is low. Nevertheless other allergen groups (especially group 1) are important causes for the high allergenic potential of pollen as well. In general, group 5 allergens are used in specific immunotherapy as a reference for allergen standardization [38]. This study provides an overview on the group 5 allergenic potential for the most important grass species / cultivars used in agriculture. For the first time a comparison between different grass cultivars and their content of major grass group 5 allergen was performed. In general, our phenological surveys and the measurement of atmospheric grass pollen concentrations indicated that the period between end of May to end of June is most problematic for people allergic to grass pollen.

The allergen content was measured by a quantitative ELISA analysis of pollen extracts which were gained from field samples. As pollen collection directly outside in the field is quite challenging and with limited success due to adverse environmental conditions, it was only possible to determine the allergenic content once during flowering period and in an assist climate chamber.

On average 0.03 ng of group 5 allergens per grass pollen grain was observed which is in good agreement with a comparable study [7] where 0.05 ng per grass pollen grain was found. The allergen content in our study varied up to 74-times within the selected cultivars demonstrating the high variability of their allergenic potential. We observed a mean mass per pollen grain of 20.20 ng whereas [7] found 11.0 ng. The average percentage of soluble protein was 12.1%. Other studies found 2%, 6% [7] and 8% [27]. Those large differences in protein content might be explained by the fact that the determination of the soluble protein content is highly dependent on the standard used in the test and thus can vary between studies.

Based on the phenological recordings carried out using BBCH code [23], the date of first flowering varied up to 25 days between cultivars, nevertheless all observed flowering dates were in well coincidence with reference values provided by the German Bundessortenamt [39]. There was no significant correlation between flowering date and cultivar-specific allergen proportions.

Provided that the detected allergenic content remains constant during the flowering period, cultivars from the species Kentucky bluegrass, italian rye grass and perennial rye grass should be grown preferentially in respect to minimizing the allergen production. Since variability in allergenic content for perennial rye grass is quite high, more cultivars from this species have to be investigated to provide solid recommendations. Due to the high allergenic content and pollen production for both investigated timothy cultivars, the cultivation of especially this species should be kept to a minimum level.

Nevertheless it is questionable whether cultivars from this species grown on intensively cultivated areas may reach flowering at all before harvesting since plant development is quite slow and thus flowering is late in comparison to most of the other species. In intensive grassland management, only a few early flowering cultivars / species, which are commonly used as indicators for sward development, come into flowering before the first silage cut, since protein content of the biomass sharply decreases when grass species flower. Consequently the local (potential) emission of grass allergens [10] and thus actual strengths of hay fever symptoms may be influenced by the time of hay cutting.

The genus bastard hybrid fescue (rye grass x meadow fescue cultivar) has on average higher soluble group 5 allergen content compared to perennial rye grass. This would mean that the allergenic content of perennial rye grass rises during the hybridization with fescue.

As grass pollen allergens are a strong trigger for hay fever and asthma [40] investigations about the allergenic potential of grasses extensively used in agriculture are absolutely necessary. Future studies are needed to analyze whether there are cultivar-specific peaks in the allergen content during plant development and thus during the grass pollen season, and to which extent meteorological conditions or respectively climate change have an impact on the allergen content.

Conclusion

The analysis of the group 5 content in different grass species/cultivars offers new insights into the allergenic risk from grasses (*Poaceae*) for patients suffering from hay fever. The study revealed that Kentucky bluegrass, italian rye grass and perennial rye grass have the least allergenic content and thus should be grown preferentially whereas timothy should be kept to a minimum.

Supporting information

S1 Table.
(XLSX)

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PLOS ONE

RESEARCH ARTICLE

Impact of elevated air temperature and drought on pollen characteristics of major agricultural grass species

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Abstract

Grass pollen allergens are known to be one of the major triggers of hay fever with an increasing number of humans affected by pollen associated health impacts. Climate change characterized by increasing air temperature and more frequent drought periods might affect plant development and pollen characteristics. In this study a one-year (2017) field experiment was conducted in Bavaria, Germany, simulating drought by excluding rain and elevated air temperature by installing a heating system to investigate their effects primarily on the allergenic potential of eight selected cultivars of the two grass species timothy and perennial ryegrass. It could be shown for timothy that especially under drought and heat conditions the allergen content is significantly lower accompanied by a decrease in pollen weight and protein content. In perennial ryegrass the response to drought and heat conditions in terms of allergen content, pollen weight, and protein content was more dependent on the respective cultivar probably due to varying requirements for their growth conditions and tolerance to drought and heat. Results support recommendations which cultivars should be grown preferentially. The optimal choice of grass species and respective cultivars under changing climate conditions should be a major key aspect for the public health sector in the future.

Introduction

Grass pollen is the major cause of aeroallergen-induced respiratory diseases [1,2]. Besides hay fever, grass pollen can also lead to severe asthmatic reactions in the lower human airways when the pollen structure is decomposed and granules are released [3].

In the last decades the length of the pollen season worldwide has been extended and just comparably, the time period in which pollen allergies occur has been prolonged [4]. These clear changes in the pollen season can be explained by an earlier flowering of plant species due to global climate warming [5,6] whereas the end of flowering seems to be largely unchanged [7]. Besides longer pollen flight seasons, the pollen production and allergenicity of pollen are influenced by higher atmospheric CO_2 concentrations [8] and an increasing number of droughts and elevated temperature [9,10]. The affected areas by such extreme conditions will increase in the next decades as well [11]. A change in the growing conditions and thus competition as well as newly invading species will also lead to a shift in the composition of species [12]. In agricultural grassland systems, extensively planted cultivars such as the early flowering variety Ivana from the species perennial ryegrass (*Lolium perenne* L.) are known to have a low tolerance level against drought due to their e.g. alpine origin with high precipitation quantities and thus their cultivation will be limited in the future. Thus, a wise selection of species and cultivars which are adapted to the altered climate conditions is necessary in order to maintain agricultural yields. It was shown that C_3 grasses in general have higher requirements regarding water availability than C_4 grasses, thus it is suggested that C_4 grasses would be more abundant when water limitation increases [13]. The change in species/cultivar composition and management such as cutting dates on agriculture land and other grassland types might also affect the pollen production and their allergenic potential [14]. Unfavorable conditions during the growing season such as extreme dry periods and heat typically reduce grass growth and might inhibit pollen production, while allergenicity potentially increases due to plant stress [15,16]. A study from Switzerland [17] showed that under the hot and dry conditions in spring and summer 2003 peaks in pollen concentration were already reached in May or beginning of June while the duration of the grass pollen season tended to be shorter than in other years. It was also found that grasses almost stopped growth and pollen production at an early stage by end of June. Accordingly patients allergic to grass pollen had severe symptoms of hay fever in May and June whereas symptoms were reduced towards the end of June.

Temperature and precipitation are the main drivers of plant growth and pollen development [18,19]. It was already shown that herbaceous taxa such as grasses are highly climate sensitive, especially for water availability, compared to other taxa [20]. A long term study [21] on grass pollination at the western Mediterranean coast revealed that elevated minimum temperatures and a rise of precipitation in spring led to higher average pollen concentrations and an earlier ending of pollen season. Other studies reported that an increase in temperature and precipitation intensifies the pollen production of early flowering species, while there is only a small effect on late flowering species [20,22], likely related to differentially impacting cutting dates [14]. The timing of water availability during the growing season also plays an important role for the plant development and affects the number of inflorescences, as it could be shown for tallgrass [23]. Nevertheless the response to the water availability during the growing season was still species-specific [23].

Under extreme growing conditions, e.g. longer drought or warm periods, plants suffer from water stress. It has already been shown that there is a clear link between plant growth and–stress of mesic temperate grasslands [13]. Whether the grass allergens are influenced by plant stress remains unclear, since their basic function inside pollen is unknown for the majority of those allergens. According to other studies the pollen release respectively pollen production is more sensitive to meteorological factors than the allergenicity [24–26]. Nevertheless for ragweed it could be shown that under elevated drought stress the expressed sequence tags (ESTs) encoding allergenic ragweed proteins increased, thus allergen content tended to increase as well [27]. Another study on Arabidopsis and rice revealed that pollen allergens tended to be part of metabolic processes in the pollen cell wall and part of stress responses [28]. These latter two studies indicate that grass pollen allergens might be enhanced under stress conditions as well.

In general, up to 95% of patients allergic to grass pollen possess IgE specific for group 1 allergens and 80% for group 5 allergens, thus these two groups make up the major grass pollen allergens [29–31]. Comparing the analysis of group 1 allergens (Phl p1) and group 5 allergens (Phl p5), the allergen quantification is much easier for group 5 allergens. Phl p1 reaches high homology in various grass species, but the immunodominant positions of the amino acids are different. In consequence the immune response to group 1 allergens might differ between grass species which makes an investigation of Phl p1 quite difficult [32].

The impact of drought and elevated temperature primary on the allergenic potential of different grass species and respective cultivars has up till now only been little examined. In this context it still needs to be clarified whether plant stress induces higher allergenic potentials in grass pollen. To quantify the impact of drought and elevated air temperature, this study conducted a one-year field experiment focusing on the effects of warming and drought on the phenological development, pollen weight, protein content and group 5 allergen content (Phl p5) of selected cultivars from the grass species timothy and perennial ryegrass. We hypothesize that dry conditions and elevated temperatures hamper pollen development and increase the allergen content due to plant stress.

Material and methods

Investigated grass species and cultivars

The following grass species and associated cultivars were selected: perennial ryegrass (*Lolium perenne* L.): Honroso, Borsato, Indra and Ivana; timothy (*Phleum pratense* L.): Comer, Lischka, Classic and the timothy grass mixture solely from the provenance Giggenhausen (48.363239˚N, 11.649388˚E); and cocksfoot (*Dactylis glomerata* L.): Musketier, Revolin, Diceros and Lidaglo. The timothy grasses from the provenance Giggenhausen are naturally-occurring grasses which are summarized in the following as one group under the name of their provenance. Due to the use of relatively old seedling material in the Lidaglo cultivar with lower germination rates and slower development in the Revolin and Diceros cultivars, the pollen production of cocksfoot was insufficient, whereby appropriate pollen amounts were produced only on the control plots by the cocksfoot cultivar Musketier. Therefore cocksfoot was excluded from the analysis since no meaningful comparisons were feasible. In the beginning of the survey, the cultivar Classic (timothy) developed more slowly in its early growing stage, i.e. seemed to be undersized and less vital, therefore it was excluded from the phenological, height and photographic recordings. Later on, Classic recovered in all treatments so that this cultivar was included into the pollen sampling and the following analytics.

Experimental site

The experimental study site (Fig 1) is located at the research station Dürnast (48.404457˚N, 11.690464˚E; 445 m a.s.l.), 50 km north east of Munich, southern Germany. The site is part of the well-maintained Gewächshauslaborzentrum Dürnast of the Technical University of Munich. In total the experimental area covered 80 m^2 in which 144 plots (each 65 x 50 cm) were installed, 36 for each of the four treatments (Fig 1 and Table 1). The original loamy soil of previous experiments [33] had been exchanged by more sandy soil material with higher drainage capacity [34]. In the beginning of 2014, this material was classified as loamier sand with a 70% proportion of sand and low phosphorus and potassium content. Before sowing of the grass cultivars in autumn 2016, 5 cm of humus was applied. For each plot 0.54 g pure seeding material from one cultivar was mixed with soya grist and then equally distributed over the respective 65 x 50 cm area. Each treatment comprised respectively four different cultivars each

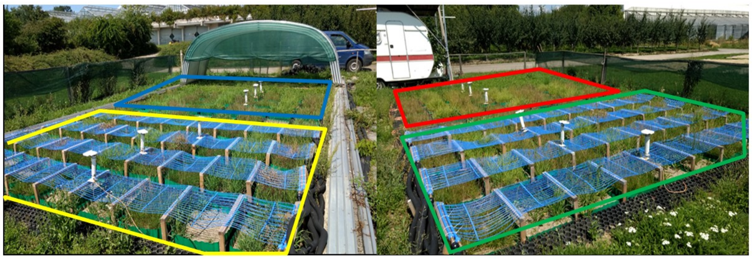

Fig 1. Experimental setup in Dürnast (48.404457˚N, 11.690464˚E). Right side: control (red) and warming treatment (green); Left side: drought treatment (blue), warming + drought treatment (yellow area).

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from the three grass species timothy, perennial ryegrass and cocksfoot (4*3 = 12 plots). Within each treatment three repetitions were conducted on a total of 36 plots (see Table 1).

Treatments

During the investigation period from May 19 until June 23, 2017, the simulation of drought (-stress) and/or elevated air temperature was conducted in three different treatments and one control (Fig 1). Besides the control, the experiment comprised the treatments drought (rain

Table 1. Experimental setup in Dürnast comprising respectively four different cultivars from the grass species timothy, cocksfoot and perennial ryegrass under three treatments and the control, three repetitions each, cocksfoot was excluded from this study since the pollen production was insufficient.

	Control				Warming				Grass species
Trailer	Comer	Giggenhausen	Lischka	Classic	Comer	Giggenhausen	Lischka	Classic	Timothy
	Musketier	Revolin	Diceros	Lidaglo	Musketier	Revolin	Diceros	Lidaglo	Cocksfoot
	Hornroso	Borsato	Indra	Ivana	Hornroso	Borsato	Indra	Ivana	Perennial ryegrass
	III. Repetition								
	Comer	Giggenhausen	Lischka	Classic	Comer	Giggenhausen	Lischka	Classic	Timothy
	Musketier	Revolin	Diceros	Lidaglo	Musketier	Revolin	Diceros	Lidaglo	Cocksfoot
	Hornroso	Borsato	Indra	Ivana	Hornroso	Borsato	Indra	Ivana	Perennial ryegrass
	II. Repetition								
	Comer	Giggenhausen	Lischka	Classic	Comer	Giggenhausen	Lischka	Classic	Timothy
	Musketier	Revolin	Diceros	Lidaglo	Musketier	Revolin	Diceros	Lidaglo	Cocksfoot
	Hornroso	Borsato	Indra	Ivana	Hornroso	Borsato	Indra	Ivana	Perennial ryegrass
	I. Repetition								
	Drought				**Drought + warming**				
Drought-Shelter	Comer	Giggenhausen	Lischka	Classic	Comer	Giggenhausen	Lischka	Classic	Timothy
	Musketier	Revolin	Diceros	Lidaglo	Musketier	Revolin	Diceros	Lidaglo	Cocksfoot
	Hornroso	Borsato	Indra	Ivana	Hornroso	Borsato	Indra	Ivana	Perennial ryegrass
	III. Repetition								
	Comer	Giggenhausen	Lischka	Classic	Comer	Giggenhausen	Lischka	Classic	Timothy
	Musketier	Revolin	Diceros	Lidaglo	Musketier	Revolin	Diceros	Lidaglo	Cocksfoot
	Hornroso	Borsato	Indra	Ivana	Hornroso	Borsato	Indra	Ivana	Perennial ryegrass
	II. Repetition								
	Comer	Giggenhausen	Lischka	Classic	Comer	Giggenhausen	Lischka	Classic	Timothy
	Musketier	Revolin	Diceros	Lidaglo	Musketier	Revolin	Diceros	Lidaglo	Cocksfoot
	Hornroso	Borsato	Indra	Ivana	Hornroso	Borsato	Indra	Ivana	Perennial ryegrass
	I. Repetition								

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exclusion by a rainout shelter), warming (elevated air temperature) and drought + warming (rain exclusion and elevated air temperature combined).

The elevation of air temperature was achieved by a micro-capillary warm water system (type P.VS30, Beka Heiz- und Kühlmatten GmbH, Berlin, Germany). The capillary mats (5,700 mm length, 630 mm width, capillary tube diameter 4.5 mm, distance between tubes 30 mm) were fixed on a wooden frame in 20 cm height. One single mat covered 9 plots in a row (Fig 1). The warming system was first installed in May 2017, when first grasses reached the height of 20 cm, in order to prevent shading effects as long as possible. In the treatment plots warming and warming + drought, the air temperature at 20 cm height above ground was increased on average by 0.87˚C during the investigation period from May 19 until June 23, 2017.

For the treatments drought and warming + drought, a transparent mobile rainout shelter simulated drought by omitting any precipitation during the investigation period (Fig 1). It was controlled by a rain sensor operating the shelter as soon as the first rain drop hit the sensor. In turn, the shelter reopened again when no further drops hit the sensor.

Plant development, soil moisture and meteorological data

After sowing in autumn 2016 plants developed well and plots were evenly covered. During the initial growing phase in 2016 and also in spring 2017 all plots were irrigated regularly by a lawn sprinkler for respectively 30 min in the morning and afternoon in order to facilitate plant development. From mid of May till end of June 2017 vegetative and reproductive phenological microstages of each plot were recorded with the expanded BBCH code on a weekly basis following [35]. Observations included all microstages between macrostage 4 (booting), 5 (inflorescence emergence, heading), 6 (flowering, anthesis), 7 (development of fruit), 8 (ripening) and 9 (senescence). The (micro-) stage was recorded for each plot on a weekly basis. Linear interpolation was used to receive the exact starting dates of flowering (BBCH 61).

The average culm height for each plot was taken in parallel to the phenological recording for each plot. On the respective harvesting dates of the cultivars, inflorescence lengths of the cut culms were measured separately for each plot.

During the investigation period digital images of each plot were taken on May 30 and June 12, 2017. The green value (DN, digital number) of each RGB image (*.jpeg) was extracted and analyzed using the package Fiji [36] which is based on ImageJ [37]. For the interpretation, green values were regarded as proxy for plant vitality.

Between May 19 and June 23, 2017, soil moisture was recorded two to three times per week in depth levels of 100, 200 and 300 mm with a soil moisture sensor (PR2 /6 SDI-12, HH2 Moisture Meter, Delta–T Devices, Cambridge, UK) at 36 spots equally distributed among the treatments where measuring tubes had been embedded in the soil. Due to very low soil water content and clear signs of dehydration of the grasses, all plots were irrigated with a watering can by 1.6 l per plot on June 01, and by 3 l per plot on June 09/14/21, 2017, respectively.

Meteorological data (air temperature, air humidity, and precipitation) in hourly resolution to characterize the growing conditions were obtained from a nearby climate station (Weihenstephan-Dürnast, location 48.4029˚N; 11.7305˚E, distance to field site 385 m) of the German Meteorological Service (DWD). In addition, air temperature and relative air humidity in 20 cm above ground were directly measured by 12 sensors at the site, equally distributed among the treatments.

Collection of grass pollen and pollen count per blade

In-situ pollen collections are very likely to be influenced by humidity (e.g. caused by rain events), mildew and insects after plants have been covered with collective containers [38]. To overcome these issues, grasses were kept in climate chambers during the actual pollen release. Accordingly, when first flowering (BBCH 61) was observed for a cultivar/treatment, bunches of 10–20 individual plants per plot were harvested and inflorescences were covered with pergamin bags and closed at the bottom. After attaining the full flowering stage (BBCH 65) under fixed conditions in a climate chamber (day/night cycle: 14 hours day at 23˚C, 10 hours night at 15˚C; air humidity 45%), pollen was extracted from the pergamin bags by shaking and subsequent removal of anthers and culms. Additionally, the number of culms per bag was counted and the total pollen weight was determined by an electronic balance (XS204DR, Mettler Toledo GmbH, Gießen, Germany). This method for pollen collection and isolation [39] was consistently applied for all samples. However, it cannot be excluded that single pollen still adhered to the bags or were not released by the anthers. To preserve pollen in the same fresh conditions before analytical testing, they were stored at -20˚C.

Extraction and determination of protein content

Following Jung et al. [38], pollen grains were mechanically extracted. Total soluble protein content was quantified using BCA test [38]. The reagents (BCA solution and copper sulfate) were ordered from Sigma (B9643; C2284) and for the standard curve Albumin from Serva (11930) was used.

Grass pollen weight and allergen quantification

Grass pollen weight was measured by dissolving 5 mg pollen grains of each grass cultivar sample immediately before the measurement in 250 μl PBS from which in turn 10μl were counted (n = 4) using an automated cell counter (TC-10, Bio-Rad Laboratories GmbH, München, Germany) [40]. All samples were counted within one day.

For the quantification of group 5 allergen content a sandwich ELISA (Allergopharma GmbH, Reinbeck/Hamburg, Germany) [41–43] was used, with a sensitivity of 1 ng/ml and precision of ±10% [38,44]. A standard curve was set up by the timothy (*Phleum pretense*) group 5 allergen Phl p5 (Allergopharma GmbH) covering a concentration range of 1 to 1000 ng/ml [38,45]. The epitopes present on the grass pollen allergens Phl p5a and Phl p5b were fixated with the monoclonal antibodies MoAb 1D11 and MoAb B01 (Allergopharma GmbH) and spectrophotometrically visualized with a chromogen present on the biotinylated MoAb B01. Since the group 5 allergens are homologous proteins in grass pollen, the same antibodies could be used for all species of *Poaceae* [46,47].

Statistical analyses

For the parameters air temperature at 20 cm height, soil moisture, green value, pollen production, pollen weight, protein content and allergen content the Shapiro–Wilk normality test was performed. Since the parameters were not normally distributed (p-value of Shapiro–Wilk test < 0.05), non-parametric tests were used. In order to compare more than two groups, the Kruskal–Wallis test was used to check for significant differences (p-value < 0.05) between the groups (e.g. temperature at 20 cm height in four treatments). Afterwards, the pairwise Wilcoxon test was chosen to identify significant differences between single pairs of more than two groups. Due to the limited number of samples for the respective treatment and cultivar, differences between treatments were tested on the species level, and not separately for each cultivar. Correlation analysis for non-parametric data was calculated by Spearman's Rank Correlation. P values smaller than 0.05 were considered to be statistically significant. All data were analyzed with R [48] using the packages ggplot2, latticeExtra, tidyr and ggpubr.

Results

Growth conditions

According to the data recorded by DWD between April 24 and July 02, 2017, the average air temperature was 15˚C and the average precipitation per day was 2.8 mm (Fig 2). There was a longer dry period between mid-May until end of May with less than 1 mm precipitation in total.

On the plots with warming system air temperature in 20 cm height above ground was on average 0.87˚C higher and relative air humidity 1.34% lower compared to the treatments without warming between May 17 and July 02, 2017 (Fig 2), but these differences between the treatments were not significant ($p = 0.325$).

Soil moisture in 100, 200 and 300 mm depth was on average 28% lower on the plots where rain was excluded by the rainout shelter ($p<0.001$). During the months May and June soil moisture in 100 mm depth was on average 0.07 m^3 m^{-3} in the warming treatment, 0.05 m^3 m^{-3} in the drought treatment, 0.06 m^3 m^{-3} in the warming + drought treatment and 0.08 m^3 m^{-3} in the control treatment (Fig 2). The minimum soil moisture of 0.04 m^3 m^{-3} was reached on June 08 for the drought treatment. Afterwards, around June 14 there was a peak in soil moisture for all treatments due to the irrigation on June 09/14/21, 2017 (see section 2.4).

Phenological development and height

Among the studied perennial ryegrass cultivars, Ivana started flowering first on May 24 (DOY 144), followed by Indra (DOY 160), Borsato (DOY 164), and Honroso (DOY 167) (Fig 3). Except Ivana, all other cultivars of perennial ryegrass tended to have a slightly faster phenological development in the treatments warming, and drought + warming ($p = 0.47$). In general, the cultivars of timothy started flowering later than perennial ryegrass. Among timothy, Giggenhausen was the first starting to flower on June 13 (DOY 164), second the cultivar Lischka (between DOY 164 and 167), third Comer (DOY 167) and latest Classic (between DOY 167 and 171) (Fig 3). Except for the drought treatment, phenological curve progression was mostly similarly among the other treatments ($p = 0.77$).

Until end of May the average height did not differ between perennial ryegrass (28.5 cm) and timothy (27.6 cm) ($p = 0.74$) (Fig 4). In the period May 17 to June 23, perennial ryegrass grew on average in height by 17.2 cm and timothy by 16.7 cm. Ivana (perennial ryegrass cultivar) had the largest total height (47.8 cm) among all investigated cultivars, whereas Borsato had the lowest total height (33.2 cm). There were no significant differences ($p = 0.75$) among the treatments w.r.t. height growth between May 17 to June 23: the control treatment had on average the strongest increase in height (19.3 cm), followed by the warming (16.6 cm), drought (16.2 cm) and warming + drought (15.2 cm), respectively. Up and downward fluctuations in the height progression can be explained by the (partial) removal of plant individuals at harvesting.

The inflorescence and spikelet length of perennial ryegrass was on average 9.1 cm and for timothy 9.6 cm. There were no significant differences between the treatments. When comparing the treatments, the largest growth in height (37.8 cm) and inflorescence length (13.5 cm) was reached under the control treatment for perennial ryegrass whereas timothy had the largest height (43.5 cm) under the warming treatment and highest inflorescence length under the drought treatment (4.0 cm). There were no significant differences between the treatments for the inflorescence length.

Pollen analytics

Weight per grain. On average the pollen weight per grain was 30% higher in timothy (18.3 ng) compared to perennial ryegrass (14.1 ng, $p = <0.01$) (Fig 5). Among the observed timothy cultivars, Lischka had the highest pollen weight (20.1 ng) and Classic the lowest (16.6

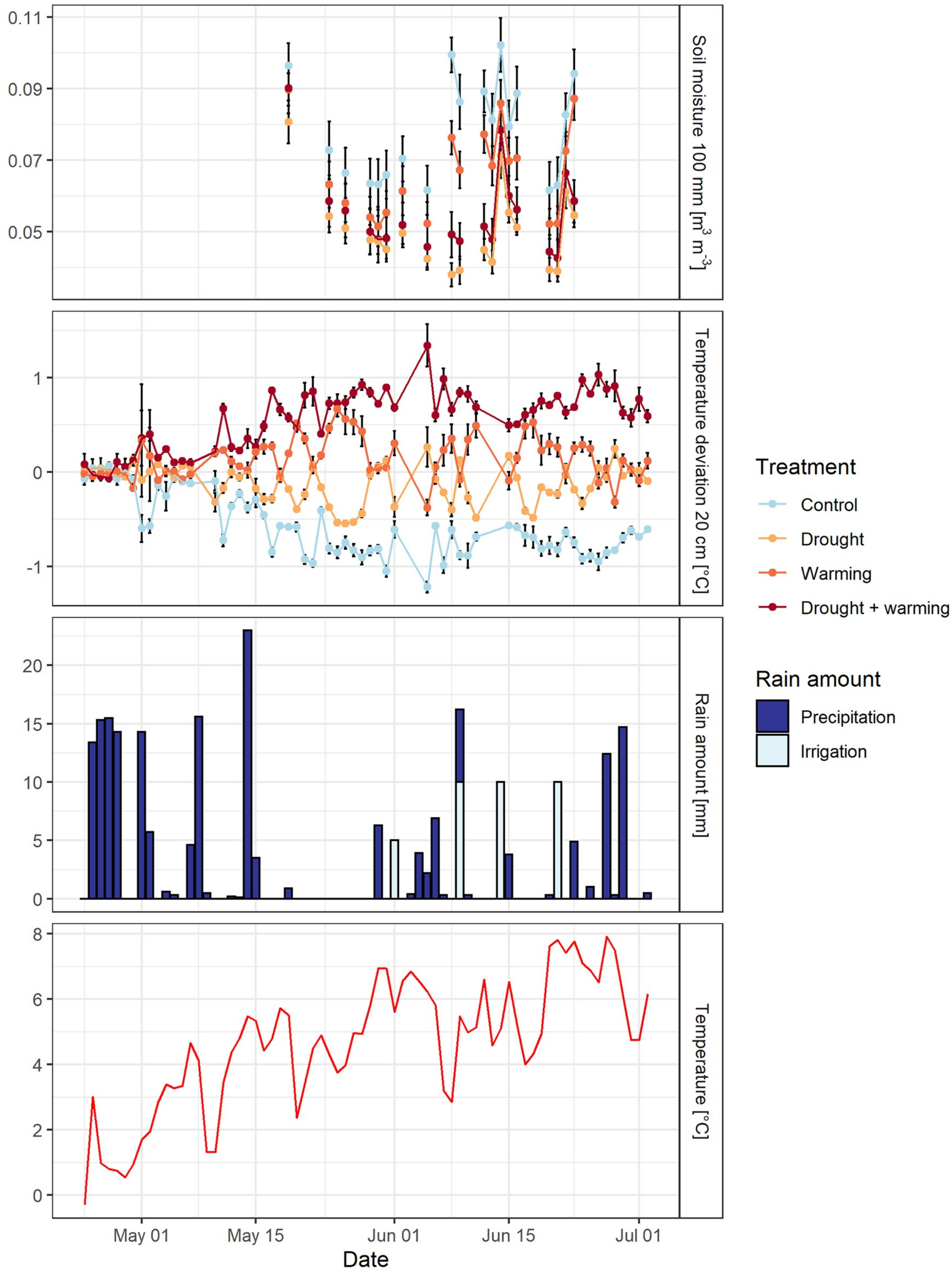

Fig 2. Meteorological parameters during the investigation period (May 17 to July 02, 2017), top to bottom: Soil moisture [m^3 m^{-3}] in 100 mm depth (measuring points were only connected for consecutive days, error bars indicate the standard error of 7–9 measurements each), mean daily temperature [˚C] as deviation from the average of all four treatments at 20 cm height (colors represent the treatments, error bars indicate the standard error of 3 measurements each), daily sum of precipitation [mm] and irrigation [mm] (in light blue), daily air temperature [˚C] obtained from the German Meteorological Service (DWD) (in red).

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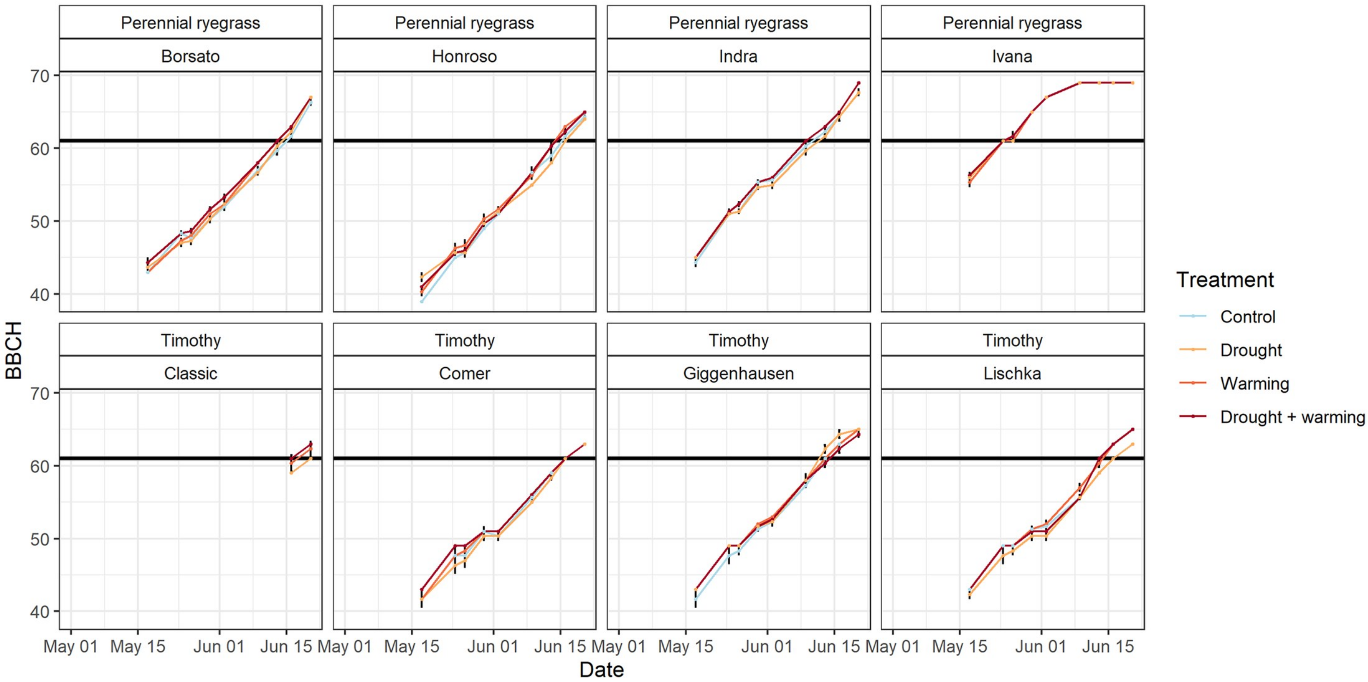

Fig 3. Phenological development of the cultivars for perennial ryegrass and timothy in spring 2017. BBCH indicates the (micro-) stages according to BBCH code [35] between May 17 and June 23, 2017. Black lines indicate the beginning of flowering (BBCH 61), parts of the data is missing for the cultivar Classic before its recovery (see Methods 2.1).

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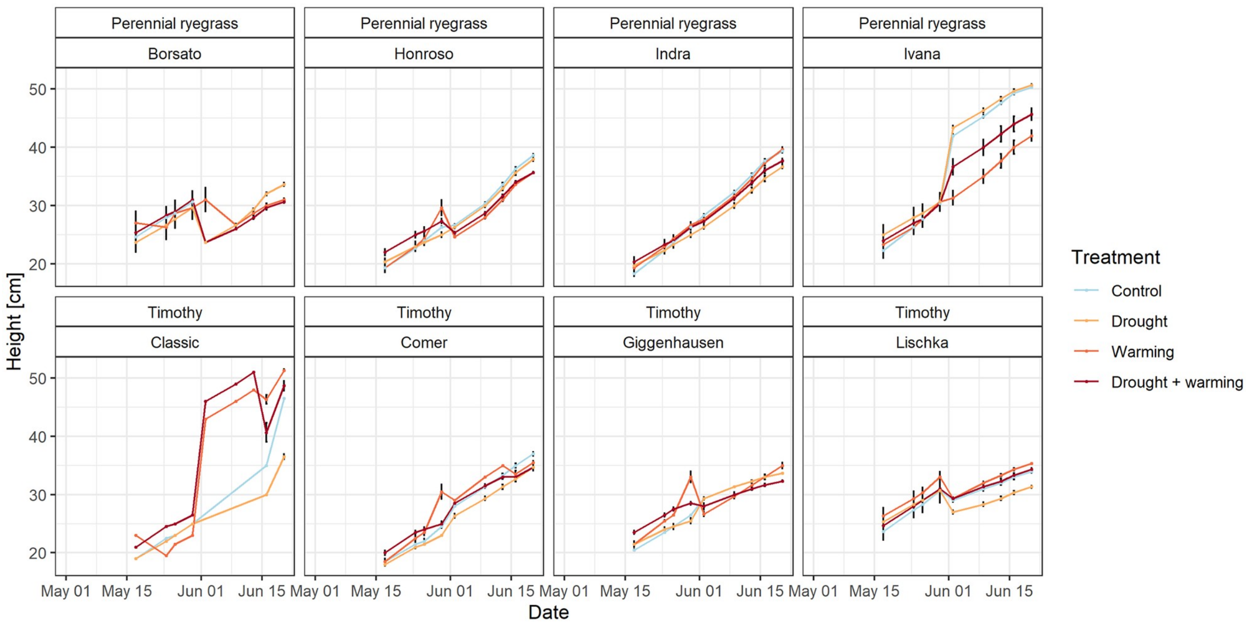

Fig 4. Height developments of the cultivars of perennial ryegrass and timothy between May 17 and June 23, 2017. Error bars indicate the standard error of three measurements each.

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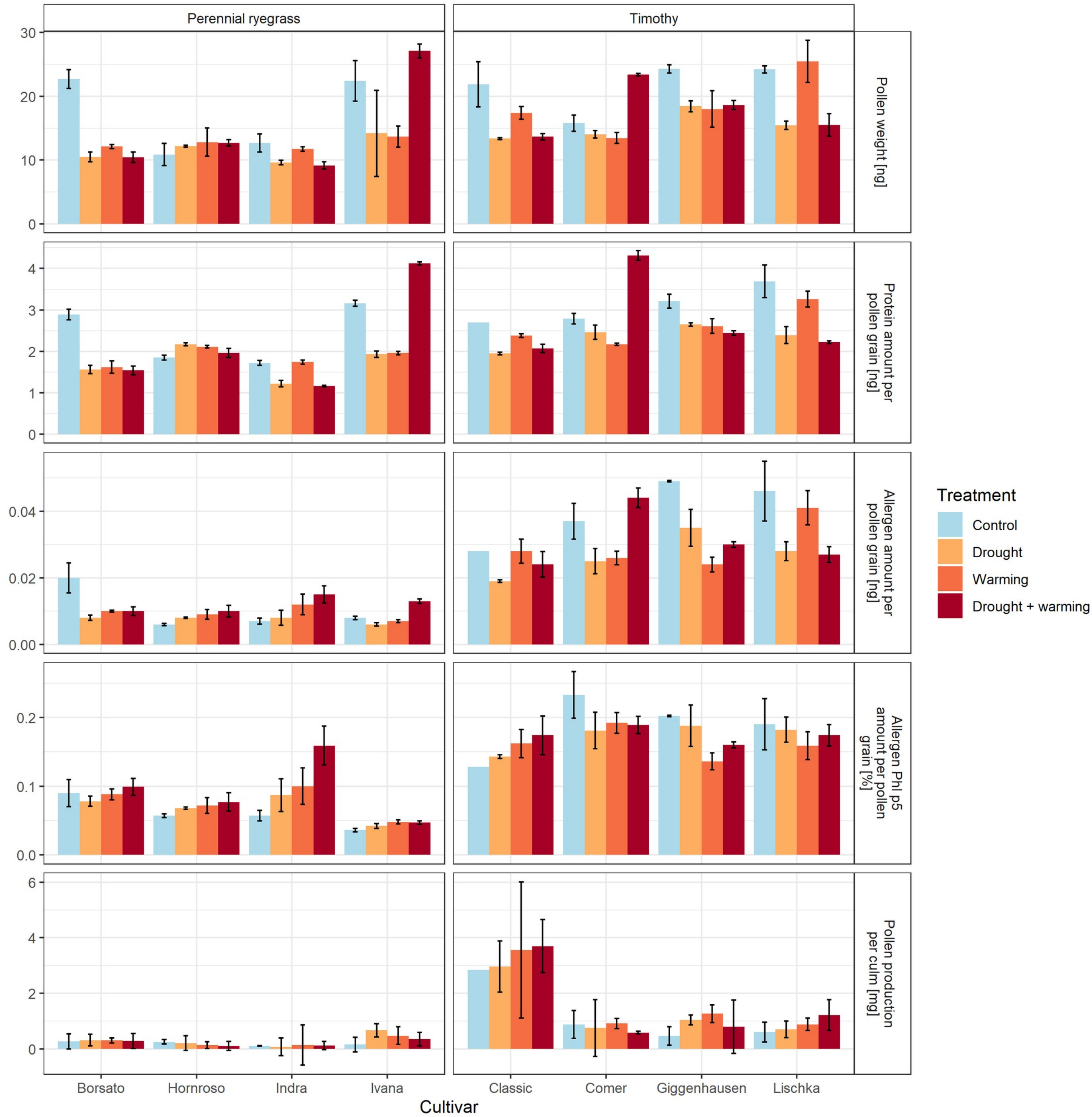

Fig 5. Pollen characteristics, top to bottom: Pollen weight per pollen grain in [ng], protein amount per pollen grain [ng], allergen amount per pollen grain [ng], allergen Phl p5 amount per pollen grain [%] and pollen production per culm [mg] for perennial ryegrass and timothy. Colors represent the treatments; error bars indicate the standard error of three repetitions (due to lack of material, Classic control was not repeated except for pollen weight).

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ng). Among the perennial ryegrass cultivars, Ivana had the highest (19.3 ng) and Indra the lowest weight per grain (10.8 ng). There were significant differences between the treatments for the perennial ryegrass cultivars ($p<0.05$) and for timothy cultivars ($p<0.01$).

Based on the limited number of samples per treatment and cultivar, statistical tests comparing treatments were carried out for all cultivars of one species combined and not separated by cultivar. For perennial ryegrass significant differences in the weight per pollen grain could particularly be seen between the control (17.2 ng) and the treatments drought (11.6 ng) ($p<0.01$), and drought + warming (12.6 ng) ($p<0.05$). For timothy significant differences were observed between the control (21.5 ng) and the drought treatment (15.3 ng) ($p<0.01$).

Protein and allergen content. The average protein amount was 32% higher in timothy (2.7 ng higher per grain) than in perennial ryegrass (2.0 ng per grain, $p = <0.01$) (Fig 5). Regardless of the species, Indra had the lowest protein content (1.5 ng) and Comer the highest (2.9 ng). Protein amount for the timothy cultivars significantly differed between the treatments control (3.1 ng) and drought (2.4 ng) ($p<0.01$). For the perennial ryegrass cultivars there was no significant effect of the treatments.

The absolute allergen content was on average more than three times higher in timothy (0.032 ng) compared to perennial ryegrass (0.010 ng) (Fig 5). The perennial ryegrass cultivar Hornroso had on average the lowest allergen content (0.008 ng) and the timothy cultivar Lischka (0.035) the highest.

The absolute allergen content (ng) was consistently and significantly higher in the control (0.040 ng) in comparison with all treatments for the timothy cultivars (average 0.029) ($p<0.01$). In the case of the perennial ryegrass there was a significant difference between the treatments drought (0.008 ng) and warming + drought (0.012 ng) ($p<0.05$).

On average the allergen proportion (Phl p5) per grain was 57% higher in timothy (0.17% per pollen grain) than in perennial ryegrass (0.08%, $p = <0.001$) (Fig 5). Among the perennial ryegrass cultivars, Ivana had the lowest (0.04%) and Indra the highest content (0.1%). For timothy cultivars, Classic had the lowest (0.15%) and Comer the highest (0.19%).

The allergen proportion (%) of perennial ryegrass cultivars was, with one exception, generally higher in the treatments warming (0.08%), drought (0.10%) and warming + drought (0.07%) than in the control (0.06%) ($p = 0.15$). For timothy cultivars the ranking was opposite, i.e. the allergen proportion for the treatments warming (0.16%), drought (0.17%) and warming + drought (0.17%) was, with one exception, lower than the control (0.19%, $p = 0.15$). However, these differences between the treatments for perennial ryegrass and timothy were not significant ($p = 0.083$ and $p = 0.156$, respectively).

Pollen per culm. Highest pollen production was observed for the timothy cultivar Classic (on average 3.26 mg/culm) and lowest for the perennial ryegrass cultivar Indra (on average 0.11 mg/culm) (Fig 5). Timothy grass produced on average much more pollen (1.44 mg/culm) than perennial ryegrass (0.24 mg/culm, $p = <0.001$).

The pollen production per culm for the perennial ryegrass cultivars were on average 25% higher in the treatments control (0.30 mg/culm), drought (0.27 mg /culm) and warming + drought (0.29 mg/culm) than in the treatment warming (0.21 mg/ culm) ($p = 0.51$) (Fig 5). For the timothy cultivars the highest production per culm was found in the warming treatment (1.65 mg/culm), followed by warming + drought (1.57 mg/culm), drought (1.36 mg/culm) and control (1.19 mg /culm), however these treatment differences were not significant ($p = 0.67$).

Green values

The green value of each plot was determined twice (May 30 and June 12, 2017) by image analysis. For the drought and warming + drought plots, the green value decreased on average by

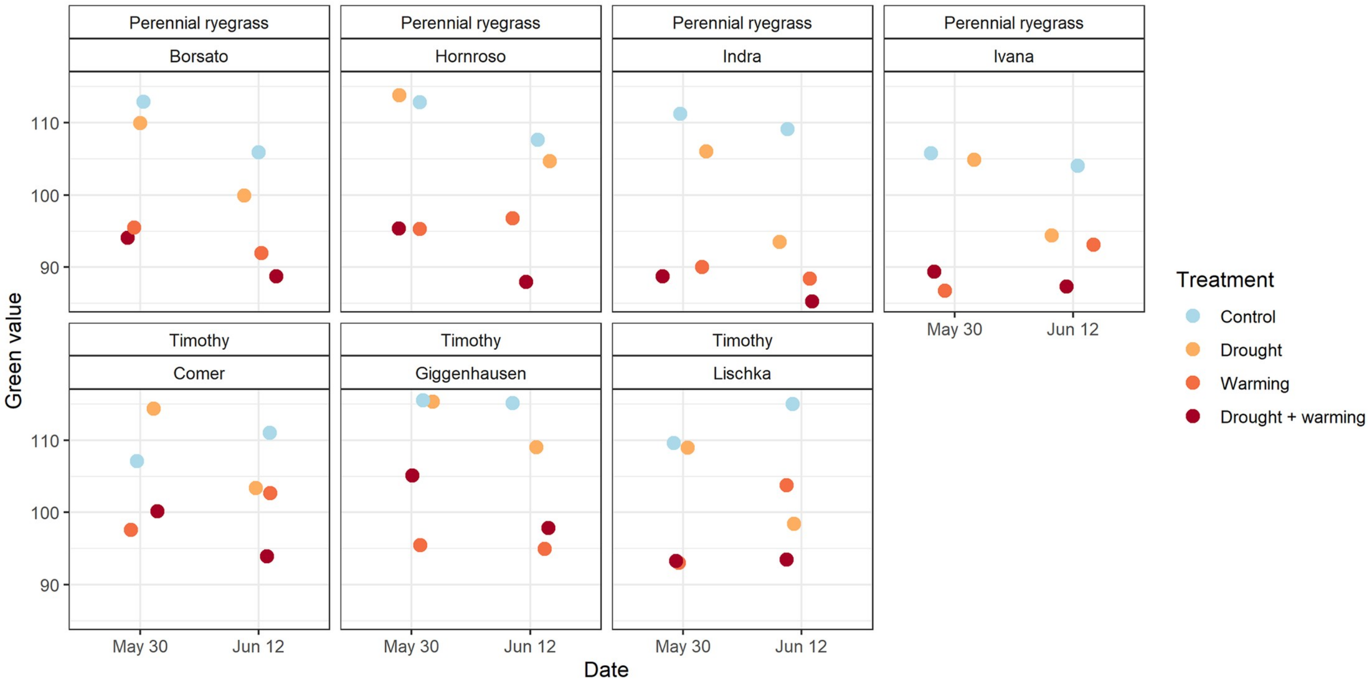

Fig 6. Green values of the cultivars from (a) perennial ryegrass and (b) timothy on May 30 and June 12 2017, the cultivar Classic (timothy) is missing because it was re-included later into the study again.

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10% and 5% respectively within these two weeks (Fig 6). In contrast the green values of the control and warming plots hardly changed, in case of the timothy cultivars Comer and Lischka the green value even slightly increased (Fig 6). Green values were generally lower (-13%) on the warming treatment plots due to the capillary mats (see Fig 1). The green values for perennial ryegrass significantly decreased between May 30 and June 12 for the control ($p<0.001$), the drought treatment ($p<0.001$) and for the drought + warming treatment ($p<0.05$). For timothy, a significant difference between green values of May 30 and June 12 could only be seen for the drought treatment ($p<0.01$).

Relationship between pollen characteristics and drought stress

The correlation analysis by Spearman between the soil moisture at all depths and the height of grasses revealed a significant positive correlation ($p<0.05$), same for the green value on June 12 ($p<0.05$) and the pollen protein content ($p<0.05$) (Fig 7). The weight per pollen grain and the protein content were highly significantly and positively correlated ($r = 0.91$, $p = <0.001$), as well as the weight per pollen grain and allergen content ($r = 0.61$, $p<0.001$), and the weight per pollen grain and the height ($r = 0.33$, $p<0.01$) (Fig 7). There was a significant correlation between pollen production per culm and allergen percentage ($r = 0.46$, $p = <0.001$) and respectively allergen content ($r = 0.44$, $p<0.001$). No significant correlation was found between the green values of May 30 and allergen content (ng) ($r = 0.21$, $p = 0.06$) whereas a significant correlation was registered for June 12 ($r = 0.42$, $p<0.001$).

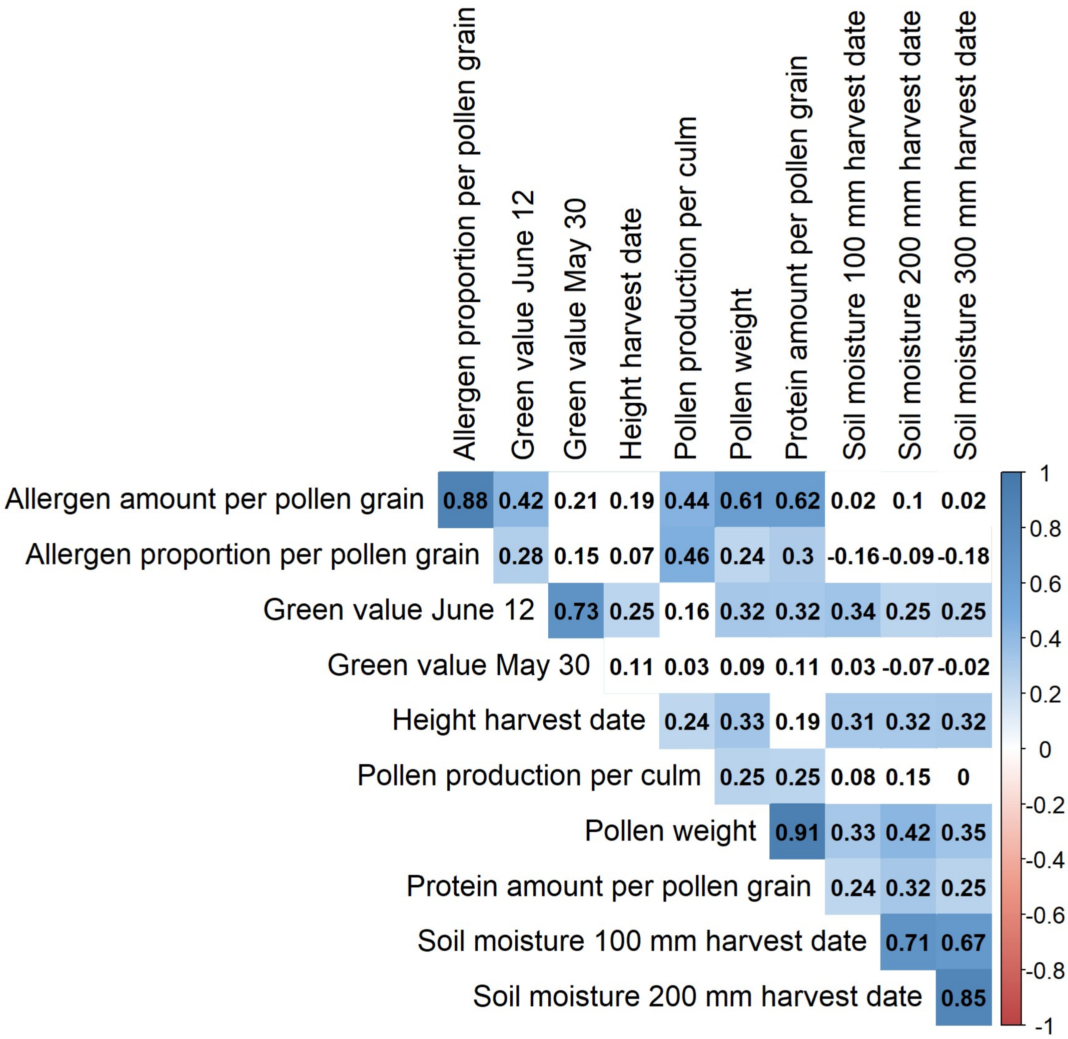

Fig 7. Spearman correlation including all treatments and the parameters allergen content (ng), allergen percentage (%), green value on May 30 and June 12, height at harvesting date (cm), pollen production per culm (mg), protein content (ng), pollen weight per grain (ng), and soil moisture in 10 cm, 20 cm and 30 cm depth at the harvest date; (level of significance 0.05, significant positive correlations indicated in blue).

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Discussion

Comparison with previous studies

Our results demonstrated that climate change related drought and warming clearly influence the development of grass in different ways e.g. by altering the start of flowering, plant development and pollen characteristics. At the same time, the recorded effects of drought and warming were strongly dependent on the grass species/cultivar.

First of all, the results of this study are in close accordance with comparable studies. The collected phenological data are similar to previous observations on plant development and the onset of flowering [38]. Although the grass individuals observed in this study are in the juvenile state, the determined ranges for pollen weight, protein- and allergen content correspond well to those of other studies [38,42]. Solely the error bars for the pollen production are relatively large due to the limited number of samples. Our results reveal significant differences between the grass species perennial ryegrass and timothy, particularly in protein and allergen contents, which are higher for timothy than for perennial ryegrass, regardless of the treatment. This result for the protein content is consistent with a previous study [49], i.e. the intraspecific variation in pollen production and allergen content is greater in perennial ryegrass than in timothy.

Grass species and -cultivar dependent response to drought and warming

Elevated temperatures slightly advance the start of flowering of grass species, perennial ryegrass and timothy. This finding supports previous studies, where elevated air temperatures also accelerated the onset of flowering similarly [2,50]. In contrast, the plant development is slightly delayed under the drought treatment with ambient temperatures [51]. The effect of drought becomes evident in the reduction of plant vitality and the markedly change in pollen development.

Pollen weight, protein- and allergen content of the timothy cultivars in drought and warming + drought treatments were in most cases significantly lower than the respective values of the control. Since the probes for pollen weight, protein and allergen content were sampled independently using different analytical methods, a systematic impact of drought on those parameters can be concluded. This systematic effect was found exclusively for timothy, but hardly for the cultivars of perennial ryegrass. The effect itself can be explained by the steadily decreasing plant vitality, which even required additional watering of all plots in mid-June. As most of the perennial ryegrass cultivars started flowering slightly earlier and in the case of Ivana clearly earlier than the timothy cultivars, pollen development was completed before plant vitality was seriously affected. This may explain why systematic effects have been observed for timothy but not for perennial ryegrass. The decrease in plant vitality was also seen in the change of green values between May 30 and June 12 on the plots where rain was excluded. During this period, the green values fell sharply and a slightly slower growth rate was recorded, clearly a sign of water shortage impacting plant development. The comparably high sensitivity of *Poaceae* to drought has already been shown by [20].

The response of the perennial ryegrass cultivars to warming/drought was in comparison to timothy more dependent on the chosen cultivar, but nevertheless a tendency towards higher allergen levels were observed under all three warming/drought treatments. For other pollen characteristics, such as pollen weight, protein and allergen content, no systematic response to the treatments was observed for the perennial ryegrass cultivars. The different reactions of the perennial ryegrass cultivars could be explained by their variable environmental constraints for growth/plant development. In consequence, their individual tolerance levels to drought or elevated temperatures could be very different. This would also explain why drought led to an increase in allergenicity in most of the perennial ryegrass cultivars, whereas a decrease was observed for most of the timothy cultivars. Specifically, the allergen proportion increased under elevated air temperatures/drought for the ryegrass cultivar Indra, Ivana and Hornroso and for the timothy grass cultivar Classic, while a decrease was observed for all other cultivars. To some extent, the influence of elevated air temperature on plant development was certainly overlaid by the interspecific variability between species and cultivars. Regarding pollen production, our results suggest that under elevated air temperatures/drought there is a high dependency on the respective cultivar.

From the climate change perspective our results propose that the allergenic risk due to timothy grass pollen will reduce in the future as the absolute allergen amount decreased under the drought treatment for all four timothy cultivars and under the warming + drought treatment for three cultivars.

The response of perennial ryegrass to drought/warming depends on the cultivar; both increases and decreases of the absolute allergen amount are possible. In comparison to timothy, perennial ryegrass has a much higher number of cultivars with 246 cultivars listed by the Bundessortenamt Germany [52,53], whereby each cultivar is highly specialized to certain environmental conditions. It has to be kept in mind that timothy had up to 5 times higher allergenic content and up to 30 times higher pollen production compared to perennial ryegrass.

Suggestions for experimental setup

Seeds for our study were obtained from provenances in Bavaria. It cannot be ruled out that specimens of the same species/cultivar but from different provenances show genetic or phenotypic variations and therefore their response to respective treatments might be different. In addition, our study is limited to a one-year field experiment and therefore can only show a portion of the perennial plant's life cycle. It could be possible that the cultivar-/ species-specific patterns shown vary within the life cycle. For example, most of the cocksfoot cultivars cultivated did not flower in the first year regardless of treatment. For validation purposes the experimental setup with the same plants was repeated in 2018. Unfortunately, due to severe technical malfunctions of the climate chambers, results from 2018 were unusable for further analysis.

Projected changes in future grasslands

Past experimental manipulations of water availability in tallgrass prairie have concluded that water limitations may be important in some years but not in others [23]. Therefore more replications of this experimental approach should be performed in several consecutive years. Due to technical prerequisites and limitations our warming treatment of 0.87˚C in 20 cm height was moderate (comparable to RCP2.6), in future stronger warming scenarios as RCP 4.5 or RCP 8.5 should be considered. The generated drought in this study simulated conditions which can be already found nowadays, also within certain areas in Germany (e.g. <30 mm precipitation in May 2017 for Brandenburg and Saxony, Source: German Meteorological Service). Dependent on the soil type and if the generated drought is stronger than in this study ($<0.04\ m^3\ m^{-3}$), the permanent wilting point ($pF < 4.2$) will be reached in certain periods, and grasses in the actual swards might not be able to develop inflorescences and emit pollen any more. Under changing climate with decreases in precipitation, the natural grass species composition will change and adapted cultivars for agricultural production have to be selected. Within the present study we show that depending on the species/cultivars a small increase in temperature and/or drought already has a high impact on the pollen allergenicity. The differences between the species-/ cultivars which revealed in some cases even opposite behavior towards drought/warming, point out the importance of grass species/cultivars selection for people allergic to pollen. In areas where e.g. fodder crops are produced agricultural aspects such as yield, quality parameters and resistance will always be prioritized, but in the development of new cultivars and release of recommendations for adjusted seed mixtures, allergenicity should be at least considered. On the other hand, in areas with extensive use such as landscape lawn or technical grassland the allergenicity of the selected cultivars should be the key factor for decision-making.

Conclusions

In this study the impact of drought and warming on pollen characteristics such as allergen amount of the two major grass species perennial ryegrass and timothy was examined.

It could be shown that the response to drought and warming is highly dependent on the species and respective cultivars, whereby both increases and decreases of the absolute allergen amount are possible, which can be explained by the cultivar specific requirements for growth conditions and tolerance to drought and heat. For the species timothy the drought and the warming treatment led to significantly lower values for pollen weight, protein content and allergen amount. In comparison, the response to drought and warming of the species perennial ryegrass was highly cultivar-specific. Based on these results, existing knowledge about different grass species and cultivars should be expanded to include the effects of drought and warming on pollen-specific traits such as allergenicity. In the long-run, the outcome of this study can contribute to the development of climate-change adapted seed mixtures which at the same time may not increase the allergenic burden. Under changing climate those aspects have to be well studied within pollen research as they are one of the key factors in public health.

Supporting information

S1 File.
(CSV)

Acknowledgments

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Supervision: Nicole Estrella, Annette Menzel.

Visualization: Stephan Jung.

Writing – original draft: Stephan Jung.

Writing – review & editing: Nicole Estrella, Michael W. Pfaffl, Stephan Hartmann, Franziska Ewald, Annette Menzel.

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Impact of Local Grasslands on Wild Grass Pollen Emission in Bavaria, Germany

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Abstract: Meteorological conditions and the distribution of pollen sources are the two most decisive factors influencing the concentration of airborne grass pollen. However, knowledge about land-use types, their potential pollen emission, and the importance of local sources remains limited. In this study, wild grass pollen concentrations from 27 stations in Bavaria, Germany, were linked to potential pollen within a 30 km radius. Agricultural grass pollen sources were derived from the InVeKos database, which contains detailed information on agricultural land-use types and their spatial distribution. Non-agricultural grassland was identified by OpenStreetMap. Further source classification was conducted using a cultivation intensity indicator and wind direction. We show that the grassland percentage and pollen concentrations, specified as annual pollen integral and pollen peak vary strongly between pollen stations. Correlation analyses indicated that the impact of the grassland on pollen concentration was greater within 10 km of the pollen traps. At greater distances, the correlation coefficient between the grassland percentage and pollen indicators steadily declined.

Keywords: aerobiology; cultivation intensity; agriculture; grassland management; pollen level; allergy

1. Introduction

Allergy-inducing pollen is the most common cause of outdoor allergies across Europe [1,2], affecting 14.8% of the population in Germany [3]. Specifically, pollen-related asthma was predicted to increase in the future due to higher sensitization rates toward pollen allergies or progressive interaction between air pollutants and pollen [4,5], although it seems to stabilize at a high level in Germany [6]. Among the pollen species triggering hay fever, those from birch and grass are the most relevant in Europe and specifically in Germany, as more than 90% of patients allergic to pollen are sensitive to these two species [7–9]. In Germany, hay fever symptoms associated with grass pollen mainly occur from May to July in parallel with the main grass pollen season. Pollen emission is highest, i.e., most grasses flower, at the beginning of June. The pollen concentration and hay fever symptoms are, to a certain degree, directly connected; thus, an increasing amount of pollen in the air induces more severe hay fever symptoms [10,11].

Around 10% of all plant species are wind-pollinated [12], i.e., their pollen is transported over short to long distances (a few meters to over 100 km) [13]. The literature provides a variety of definitions for short-, mid-, and long-distance pollen transport without differentiating between species. Sofiev and colleagues [14] suggested the following three different scales for pollen transport: micro- and local-scale (meters to kilometers), regional (up to around 100 km), and long-range (>100 km) transport. The transport distance of pollen strongly depends on the release height and the pollen size, weight, and shape. Compared to other wind-pollinated plant species, grass pollen has considerably larger grain sizes (20–55 µm) [15] and its height of release is low; both factors reduce the possibility of long-distance atmospheric transport and instead facilitate transport at local and regional scales (up to 100 km) [16–19]. This also explains why the grass pollen concentration differs more in the first 10 m above ground than birch pollen [20]. Due to the abundance of widespread vegetation up to 1500 m above sea level (a.s.l.), the pollen concentration remains mostly unchanged and only decreases at higher altitudes [18,21].

Plant phenology and the transport of airborne pollen depend largely on meteorological parameters, such as air temperature, precipitation, air humidity, and wind speed/direction [22]. These parameters, in addition to atmospheric CO_2 concentration [23], precipitation in spring [24], and land use/management [25], also have a strong impact on the amount of pollen being released.

Furthermore, the grass pollen concentration and composition are strongly influenced by the spatial source distribution [26,27]. In turn, the source distribution is strongly affected by land use [28,29] and changes in land use [30]. The land area of Germany is predominantly used for agriculture and forestry (87%); built-up areas occupy 9% of the area [31], and there is a small amount of completely unused land (<1%) [31]. The type of land use crucially determines the habitat composition, biodiversity, and processes in the ecosystem [32]. The potential area for grass pollen release is much smaller in urban than rural areas [33–38]. For cities, sources for grass pollen are mainly found in parks, private dormitory gardens, and roadside greenery, which can be classified as non-agricultural grassland. Rural areas are predominantly covered by farmland and forest. In forests, potential grass pollen sources occur only at the edges and in recently afforested/reforested areas. Agricultural grasslands, as the main potential source for grass pollen, can be roughly divided into three basic types of grassland as follows: arable land growing cereals, pastures, and meadows. Although cropland accounts for the largest proportion of the land area, most of the cereals grown there do not emit pollen (wheat—*Triticum aestivum*) or they are too large and heavy to be transported over long distances (maize—*Zea mays*), so that only a very small proportion of cereal pollen (e.g., rye—*Secale cereale*, which is rarely grown near the pollen stations) is transported over distances of more than 500 m [39]. In comparison, emitted pollen from pastures and meadows are much smaller and lighter and can thus be transported further.

The frequency and timing of cutting determines whether grasses reach maturity (the flowering stage) and are able to release pollen [25]. On pastures, the potential pollen release is determined by the number of animals per surface unit, animal species, and duration of grazing. Most agricultural grasslands are intensively cultivated throughout the year, resulting in many cuts (up to six times). As a result, the majority of agriculturally cultivated grasses do not flower, and only species with very early flowering dates, such as meadow foxtail (*Alopecurus pratensis)*, emit pollen. In contrast, on uncultivated land or land used at low intensity, the probability of flowering is much higher. It has been assumed that most species do not flower when cut at least twice per year [40].

Agricultural financial funding programs by the state entail a limitation of cuts per year and a strict regulation of the timing of the first cut. In Bavaria, Germany, Bayerisches Kulturlandschaftsprogramm (KULAP) and contractual nature conservation (Bayerisches Vertragsnaturschutzprogramm, VNP) are the most important programs for management in cultural landscapes hosted by the state. These programs outline the conditions under which agricultural holdings receive financial subsidies in return for applying specific measures to their land. This entails, among other conditions, the establishment of grassland on sensitive areas (KULAP), non-utilization of biotope category meadow, and the delay of the first cut (VNP). Considering high cutting frequencies in managed grasslands, attention should be paid to the field margins and fallow areas where the cutting frequency is predicted to be low. Another decisive factor for the pollen concentrations/pollen deposition at a site is its distance from the source and the distribution of sources in the surroundings.

The dispersal of cereal pollen around cultivated fields is strongly influenced by harvesting activities [41]. In another study, pollen release from grasses was found to have declined over the last century due to changes in land use accompanied by higher cultivation intensity [42]. In a study that looked at the impact of green spaces (arboreal species), it was shown that the pollen dispersal is strongly dependent on species and that pollen concentrations are unevenly distributed in urban areas [43]. In contrast to these studies and other well-investigated aspects, little is known about how wild grass pollen concentration is affected by the type of land use and management intensity. The main research aim of our study was to investigate the relationship between the pollen concentrations of wild grasses and potential pollen sources, taking into account distance and management intensity. Grass pollen concentrations were measured in daily resolution at different locations in Bavaria, Germany in 2015. The analysis included plotting the distance-based relationship between pollen concentration and distribution of potential pollen sources outlining spatial differences with land use/management.

2. Materials and Methods

2.1. Pollen Data

In 2015, atmospheric concentrations of pollen from several plant taxa were measured at 25 stations throughout Bavaria, Germany as part of the Elektronisches Polleninformationsnetzwerk (ePIN) project [44]. Respective data from two additional pollen stations were used (see Figure 1 and Table 1), one run by TUM in Freising (DEFREI, 48°40′05″ N, 11°71′81″ E) and one by TUM/UNIKA-T in Augsburg (DEAUGS, 48°32′60″ N, 10°90′30″ E).

The pollen concentrations were recorded by Hirst-type pollen samplers, in which airborne particles were continuously collected on adhesive tape, and pollen counts were then examined manually under the microscope following guidelines of the European Aerobiology Society [45]. The pollen dataset comprised daily pollen concentrations in grains m^{-3} of 13 selected pollen taxa, but in this study, we focus exclusively on wild grass pollen from the *Poaceae* family. The grasses studied here only include wild grasses (cultivated and uncultivated), excluding cereals, unless specifically mentioned otherwise. In Bavaria, the most common species on permanent grassland are: perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum*), cock's-foot grass (*Dactylis glomerata*), Kentucky bluegrass (*Poa pratensis*), rough bluegrass (*Poa trivialis*), meadow foxtail (*Alopecurus pratensis*), meadow fescue (*Festuca pratensis*), timothy (*Phleum pretense*), yellow oatgrass (*Trisetum flavescens*), tall oat grass (*Arrhenatherum elatius*), red fescue (*Festuca rubra*), dog's tail grass (*Cynosurus cristatus*), couch grass (*Agropyron repens*), bentgrass (*Agrostis spp.*), velvet grass (*Holcus lanatus*), soft brome (*Bromus hordeaceus/mollis*), and tufted hairgrass (*Deschampsia caespitosa*) [46]. The Hirst-type pollen samplers were placed approximately 10–20 m above ground level (a.g.l.). It has been reported that, due to dispersal dynamics, the grass pollen concentration on the ground can be up to twice the measured values at the pollen trap due to dispersal dynamics, but this also depends on the abundance of vegetation in the vicinity [20,47].

Table 1. Information about the 27 pollen stations and their respective average temperature (T) and total precipitation (P) during grass pollen season from the closest DWD weather stations. The potential source area (PSA) of grass pollen according to Oteros and colleagues [44] as grassland area within 30 km circles [%] is given, weighted by the overall cultivation intensity (CI) and wind direction for 23 selected stations in 2015 ($PSA_{CI+wind}$). DEBERC, DEHOF, DEGARM, and DEUFS are not analyzed as their immediate vicinity to the German border results in a lack of data on CI.

Station ID	Station Name	Altitude	T	P	Overall Ring, Averaged $PSA_{CI+wind}$
		[m a.s.l.]	[°C]	[mm]	
DEALTO	Altötting	398	19.7	37.6	5.68
DEAUGS	Augsburg	497	16.8	164.9	6.09
DEBAMB	Bamberg	238	18.0	127.9	7.48
DEBAYR	Bayreuth	419	15.7	104.9	9.72
DEBERC	Berchtesgaden	573	15.9	125.5	–
DEBIED	München-Biederstein	510	19.4	276.0	8.67
DEDONA	Donaustauf	425	17.2	147.9	6.41
DEFEUC	Feucht	365	17.8	140.8	6.80
DEFREI	Freising	448	18.1	213.3	11.13
DEGAIS	Gaissach	717	17.1	458.9	15.56
DEGARM	Garmisch-Partenkirchen	821	16.0	130.2	–
DEHOF	Hof	531	16.5	146.0	–
DEKITZ	Kitzingen	246	16.1	15.9	4.64
DEKOES	Kösching	391	18.6	120.4	5.87
DELAND	Landshut	397	18.7	212.5	5.24
DEMARK	Marktheidenfeld	216	18.1	93.0	7.01
DEMIND	Mindelheim	610	17.3	359.6	13.38
DEMUNC	München_Thalkirchen	538	19.2	258.8	8.20
DEMUST	Münnerstadt	347	16.0	8.1	9.02
DEOBER	Oberjoch	870	16.3	409.5	19.57
DEOETT	Oettingen	431	17.8	178.4	7.18
DEPASS	Passau	318	18.9	276.3	6.46
DETROS	Trostberg	483	18.2	375.1	9.00
DEUFS	Schneefernerhaus	2650	5.9	43.5	–
DEVIEC	Viechtach	459	14.4	166.7	9.41
DEWEID	Weiden	403	17.4	142.2	5.09
DEZUSM	Zusmarshausen	483	17.7	292.7	5.63

The pollen season was defined by applying the moving average method proposed by Rojo and colleagues [48] on the annual times series of daily pollen concentration. A threshold of 5 pollen grains m^{-3} was set by default to determine the start and end of pollen season. The Annual Pollen Integral (API) [49] was then calculated by summing the daily concentration over the pollen season. The pollen peak (PP) [50] was defined as the highest daily pollen concentration.

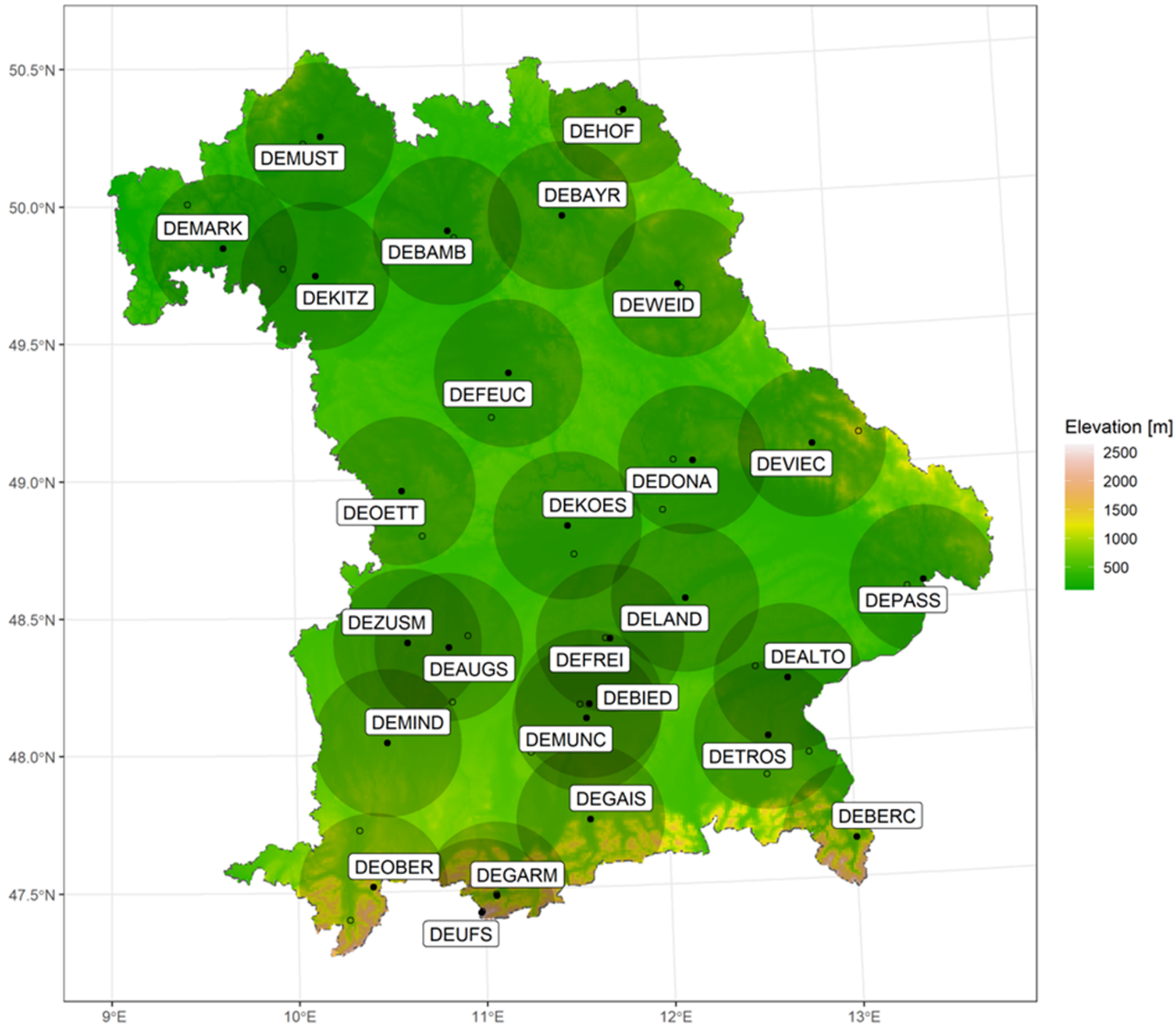

Figure 1. Locations of the 27 pollen stations in Bavaria, Germany with 30 km surrounding concentric rings (60 concentric rings in 500 m steps up to a 30 km radius (see Section 2.3)). The corresponding weather stations are marked by open circles. The background map is filled with Digital Terrain Model Grid Width 1000 m (DGM1000, GeoBasis-DE/BKG 2020, dl-de/by-2-0, http://www.bkg.bund.de, accessed on 31 December 2021). The full names of the stations and their exact locations are listed in Table 1.

2.2. Grass Pollen Sources

Grasslands included both agricultural grass pollen sources (pastures and meadows) and non-agricultural pollen sources (parks, open green spaces, private gardens, and wild gardens). Pollen sources from arable land have their own specific characteristics compared to wild grass pollen sources. From the wind-pollinated species, the cultivation of maize predominates the arable land in Bavaria. In comparison, rye constitutes a very small total share. Since cereals have low pollen emissions that are nearly impossible to capture in the pollen traps used (due to their large diameters [maize 91–93 µm] [51] and high pollen weight), arable lands with cereals were excluded from this study.

2.2.1. Area of Agricultural Grassland Derived from InVeKos

In Bavaria, 46.6% of the total area is agriculturally cultivated, 89.5% of which is held by family-run businesses [52]. Information on the spatial distribution and type of cultivation within agricultural areas is provided by the InVeKos database (Integriertes Verwaltungs- und Kontrollsystem) which was introduced by the European Commission in 1992 and is valid for all EU member states. InVeKos was set up to regulate and control a uniform agricultural policy within the EU member states. The land-use data derived from InVeKos have a high spatial resolution of 1 m and are at the cadastral unit level. The available InVeKos data are limited to the area of Bavaria. All potential emission sources within the Bavarian borders for wind-pollinated grass pollen were included in the analysis (see Table 2).

Table 2. (**A**) InVeKos and (**B**) OpenStreetMap land-use types containing grassland ordered by their respective proportions of the total grassland area within 30 km circles (German boundary) at all 23 selected pollen stations (DEUFS/DEGARM/DEBERC/DEHOF excluded). Within InVeKos land-use types, the proportions of cultivation intensity CI (i.e., <0.3 low intensity, 0.3–1.4 moderate, and ≥1.4 intensive) are based on RGV/HFF. Within OpenStreetMap land-use types, the proportions of cultivation intensity (0 = extensive; 0.3 moderate, and 1.4 intensive) are based on manual inspection of CI values (see Section 2.3). Descriptions are made based on the information available from the two databases.

(A) InVeKos

Land-Use Type	Proportion	Description	<0.3 CI	0.3–1.4 CI	≥1.4 CI
451	54.33	Meadow (including meadow orchard)	16.47	23.97	59.56
452	16.80	Mowing pasture	2.80	51.98	45.22
453	2.88	Pasture	6.97	64.31	28.72
62	2.52	Fallow (ecological compensation conservation areas)	64.50	12.77	22.73
455	2.12	Alpine pasture	41.77	56.45	1.79
441	1.86	Newly sown grassland	44.71	24.69	30.60
424	1.55	Agricultural grassland (due to crop rotation)	22.09	17.82	60.09
460	1.30	Sheep grazing	2.89	95.88	1.23
458	0.74	Litter meadow	16.57	63.27	20.15
560	0.66	Arable land; set aside (due to agri-environmental schemes)	77.65	14.20	8.15
592	0.41	Permanent grassland; set aside	81.81	10.30	7.89
930	0.35	Fish farming pond area	74.17	14.14	11.70
591	0.34	Cropland; set aside	82.25	11.46	6.28
941	0.22	Fallow under organic farming (permanent grassland)	80.00	17.94	2.06
454	0.21	Rough pastures	3.88	90.63	5.49
852	0.12	*Miscanthus* (Silvergrass)	70.10	15.07	14.83
58	0.11	Field margin (ecological compensation conservation areas)	59.18	10.29	30.53
853	0.11	Tall Wheatgrass Cultivar Szarvasi-1 (*Elymus elongatus* ssp. *ponticus* cv. *Szarvasi-1*)	67.29	18.81	13.90
803	0.09	Sudangrass (*Sorghum* × *drummondii* or *Sorghum sudanense*)	56.16	28.66	15.18
912	0.09	Seed production for grasses according to Seed law	72.79	12.73	14.48
958	0.09	Nature conservation area (without agricultural management)	61.95	27.53	10.51
428	0.06	Temporary grassland	19.62	13.76	66.62
429	0.04	Other fodder plant	49.04	28.52	22.44
56	0.04	Buffer strips along cropland (ecological compensation conservation area)	60.79	7.87	31.34
870	0.03	Energy crop in mixed culture	55.66	36.38	7.96
940	0.03	Uncultivated pond area	80.59	6.39	13.01

Table 2. *Cont.*

(A) InVeKos					
Land-Use Type	**Proportion**	**Description**	**<0.3 CI**	**0.3–1.4 CI**	**≥1.4 CI**
54	0.01	Buffer strips along woodland edges (ecological compensation conservation area)	34.45	11.89	53.66
546	0.01	Inoperative permanent grassland (FELEG)	99.65		0.35
567	0.01	Inoperative permanent grassland (agri-environmental measures)	53.20	30.47	16.33
57	<0.01	Buffer stripe (ecological compensation conservation areas, permanent greenspace)	40.42	9.11	50.47
835	<0.01	Other nuts		100	
854	<0.01	Ribbon grass	39.39		60.61
920	<0.01	Home or kitchen garden (without agricultural use)	71.59	13.88	14.53
(B) OpenStreetMap					
Land-Use Type	**Proportion**	**Description**	**0 CI**	**0.3 CI**	**1.4 CI**
Meadow	7.94	Meadow, possibly used for grazing cattle		100	
Grass	1.76	Areas with grass		100	
Park	1.61	Park			100
Allotments	0.56	Small private gardens			100
Graveyard	0.37	Cemetery or graveyard			100
Recreation ground	0.34	Open green space for general recreation		100	
Heath	0.30	Heath areas	100		

InVeKos does not include any further information on the management intensity, e.g., the number of harvests per year, which depends on the respective agricultural holding and is strongly driven by regional differences [53]. Instead, we used the "RGV/ha HFF" variable, established to financially reward agricultural holdings for limiting their cultivation intensity, as an indicator for the management intensity. RGV/ha HFF is defined as the number of cattle per hectare fodder (RGV = fodder-consuming livestock unit equivalent to 500 kg live weight) divided by the sustaining area (HFF = permanent grassland, silage maize, and arable fodder). For simplicity, we call this variable cultivation intensity (CI). Smaller numbers denote to a lower RGV/ha HFF with correspondingly fewer harvests per year, indicating low-intensity land use, whereas larger numbers refer to a higher RGV/ha HFF with correspondingly more harvests per year, indicating intensive use. Specifically, based on the Bayerisches Kulturlandschaftsprogramm (KULAP) and contractual nature conservation (Bayerisches Vertragsnaturschutzprogramm, VNP) (http://www.stmelf.bayern.de/Foerderwegweiser (accessed on 10 January 2022)), 0–0.3 RGV/ha HFF refers to grassland under no/incomplete use, 0.3–1.4 RGV/ha HFF to low-intensity use, and greater than 1.4 RGV/ha HFF to intensive use. Each agricultural holding receives its own CI, which provides detailed insight into the area-specific cultivation intensity. All selected grasslands could be assigned to one of the three CIs (<0.3, 0.3–1.4, and >1.4; see also Table 2).

2.2.2. OpenStreetMap

As the InVeKos data set comprises solely agricultural grassland, non-agricultural grasslands were identified by OpenStreetMap (Data/Maps Copyright 2018 Geofabrik GmbH and OpenStreetMap Contributors). OpenStreetMap is an international project founded in 2004 with the goal of providing a high-resolution, freely accessible map of the world. It comprises both non-agricultural and agricultural grassland; therefore, InVeKos and OpenStreetMap were overlaid in order to identify non-agricultural grassland from OpenStreetMap.

In contrast to the InVeKos data set, there is no RGV/ha HFF for grasslands available in OpenStreetMap. Therefore, the cultivation intensity was assigned by manual inspection for the different land-use types. Parks, small private gardens, and graveyards were considered intensive use, so they were assigned to CI 1.4; meadows, grasslands, and recreation grounds with mostly low-intensity use were assigned to CI 0.3; and unused heath was assigned to CI 0 (Table 2, lower part).

All InVeKos/OpenStreetMap land-use data were processed in ArcGIS (Esri 2019. ArcGis Deskop: Version 10.6.1. Redlands, CA, USA) as vector data in EPSG:32632 projection. The ring buffers were also created in ArcGIS.

2.3. Potential Source Areas for Grassland Pollen Emission

The data on agricultural grasslands from InVeKos were only available to us for Bavaria; thus, the 30 km surroundings of each pollen trap were cut off at the Bavarian border. The grasslands from the neighboring states of Austria, Czech Republic, and other German federal states were not considered. Thus, pollen stations DEBERC, DEHOF, DEGARM, and DEUFS had to be excluded from the final analysis, as large proportions (>50%) of their 30 km surrounding areas were outside the Bavarian state territory.

We applied the concentric ring method (CRM) by Oteros and colleagues [44] to study the correlations between the pollen concentrations and the respective potential emission surfaces for wild grass pollen (*Poaceae* family).

Potential source area is given by Equation (1):

$$PSA = \frac{\sum_1^n A_{grassland,\ i,\ n}}{A_{ring,\ i}},\ i = 1, \ldots, 60, \tag{1}$$

where A denotes the covered area size, i denotes the ith concentric ring from the station center till 30 km surroundings per 500 m, and n denotes the nth grassland area inside the concentric ring. Based on the exact geographical coordinates of all selected 23 pollen stations, 60 concentric rings of 500 m width were placed around each station. The maximum radius of 30 km was chosen in order to avoid greater overlap between the stations (see Figure 1).

Potential source area weighted by cultivation intensity is given by Equation (2):

$$PSA_{CI} = \frac{\sum_1^n \left[A_{grassland,i,n} / \left(CI_{i,n}{}^3 + 1\right)\right]}{A_{ring,i}},\ i = 1, \ldots,\ 60, \tag{2}$$

where $CI_{i,n}$ denotes the cultivation intensity CI (RGV/ha HFF) for each grassland area. The CI weighting of the grassland percentage was conducted by summing the RGV/ha HFF weighted grassland areas in each ring, followed by division of the sum by the whole ring area.

Potential source area weighted by cultivation intensity and directional wind frequency is given by Equation (3):

$$PSA_{CI+wind} = \frac{\sum_{i=1}^4 (\omega_i * PSA_{CI+wind,i})}{\sum_{i=1}^4 \omega_i}, \tag{3}$$

where ω_i denotes the wind frequency in each quarterly direction (NE, NW, SE, and SW) based on hourly wind measurements during the specific pollen season period (see Section 2.1) at respective pollen stations. For the combined CI and wind direction weighting, the rings were divided into four quarters. Each quarterly ring was calculated separately by dividing the pollen source area and the corresponding quarter area of the respective ring, resulting in 240 different values for each pollen station. Hourly wind speed and wind direction data for 2015 were taken from the closest DWD weather station (distances vary from hundreds of meters to around 40 km) with the same sampling height of 10 m a.g.l.

Afterwards, Pearson correlations were calculated between API/PP and PSA/PSA_{CI}/$PSA_{CI+wind}$ for each ring separately, taking into account all 23 selected pollen stations. The correlation coefficients at different distances (0–30 km) were determined in order to observe the concordance between the pollen concentration and potential source area indices.

All the data processing and analyses were performed under R [54]. Pollen-related indices were calculated by the R package AeRobiology [42]. Data visualization was performed using R package ggplot2 [55].

2.4. Sensitivity Analysis

The OpenStreetMap non-agricultural grassland has an average share of 25.6% of the overall CI weighted PSA_{CI}. It is therefore considered to have an important impact on the pollen concentration. Due to its low management intensity and generally late cutting, the measured pollen concentrations could be strongly influenced by this non-agricultural grassland. For the following sensitivity analysis, the grassland percentage from OpenStreetMap was weighted based on the registered airborne pollen concentrations instead of the land use-based CI, whereas the weighting of grassland percentages from InVeKos database remained unchanged. More specifically, for this approach, the pollen stations were divided into three groups according to their API levels (highest third, middle third, and lowest third). In the next step, the grass percentages from OpenStreetMap were weighted station-wise and the following CIs were assigned according to API levels: high API = CI 0, middle API = CI 0.3, and low API = CI 1.4. In the next step, the correlation coefficients based on grassland percentages before and after sensitivity CI weighting were subtracted.

3. Results

3.1. Grass Pollen Calendar

The grass pollen season in 2015 for all of Bavaria lasted from 30 April (e.g., at stations DEMARK, DEOETT, DEPASS, DEVIEC, and DEZUSM) until 5 September (at station DETROS), with large differences in the lengths of the pollen season and peak values among pollen stations (see Figure 2). The total grass pollen season reached up to 127 days at station DETROS, while station DEUFS exhibited the shortest pollen season of only 10 days. Alpine station DEUFS at 2650 m a.s.l. has no grass vegetation in its surroundings. The pollen measured during its extremely short pollen season is due to transport processes, as has been demonstrated for other pollen species [56]. Compared with the onset (30 April–30 May), there was higher variability in the end of the season (8 June–5 September).

The PP was reached between 29 May (DEAUGS/DEGAIS/DELAND) and 8 June (DEBAYR), with most peak dates on 3 June (12 of 27 stations). The average pollen concentration on the peak day was 288 pollen grains m^{-3}, the lowest peak pollen concentration was 28 pollen grains m^{-3} (DEUFS and DEALTO), and the highest peak pollen concentration was 618 pollen grains m^{-3} (DEOETT).

Similarly, those pollen stations with low/high peak values showed a low/high API, as follows: DEALTO (67 pollen grains m^{-3}), DEUFS (72 pollen grains m^{-3}), DEMUST (148 pollen grains m^{-3}), and DEOETT reached the highest annual pollen sum of 5992 pollen grains m^{-3} (see Figure 2). On average, an API of 3056 pollen grains m^{-3} was derived for all 27 stations. DEMUST, DEUFS, and DEALTO did not show any days when pollen concentration exceeded 100 pollen grains m^{-3}; in contrast, DEAUGS had 14 such days.

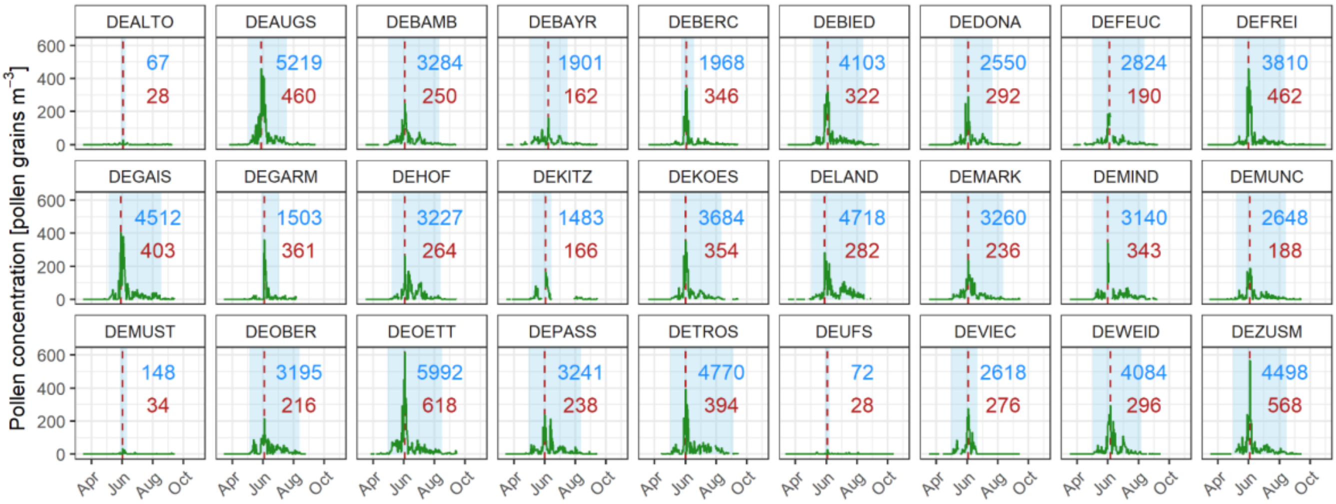

Figure 2. The grass pollen calendar of the 27 pollen stations for 2015 in Bavaria, Germany. The time series of daily pollen concentrations are shown with information on the annual pollen integral (API, in blue shades and numbers) and pollen peak (PP, in red dashed vertical lines and numbers) for each station.

3.2. Potential Pollen Source Areas $PSA_{CI+wind}$

The occurrence of grassland in Bavaria increases in areas with cooler average temperatures, increasing precipitation and with higher altitudes (multiple linear regression, $p < 0.0001$, adj. R2 66.2%). For all 23 selected pollen stations, an overall mean (weighted) $PSA_{CI+wind}$ of 8.4 was calculated. The station/ring $PSA_{CI+wind}$ values are shown in Figure 3. The highest potential source areas for grasslands were found at pollen stations DEOBER, ranging from 30 to 45 in the closer ranges (0–15 km), followed by DEGAIS (26.40 at 4 km, 25.85 at 1 km) and DEFREI (around 25 at 1.5–2.5 km). In contrast, the lowest $PSA_{CI+wind}$ values were observed at DEMIND (0.97 at 500 m), DEALTO (1.34 at 1 km, 1.64 at 500 m), and DEKOES (1.91 at 500 m).

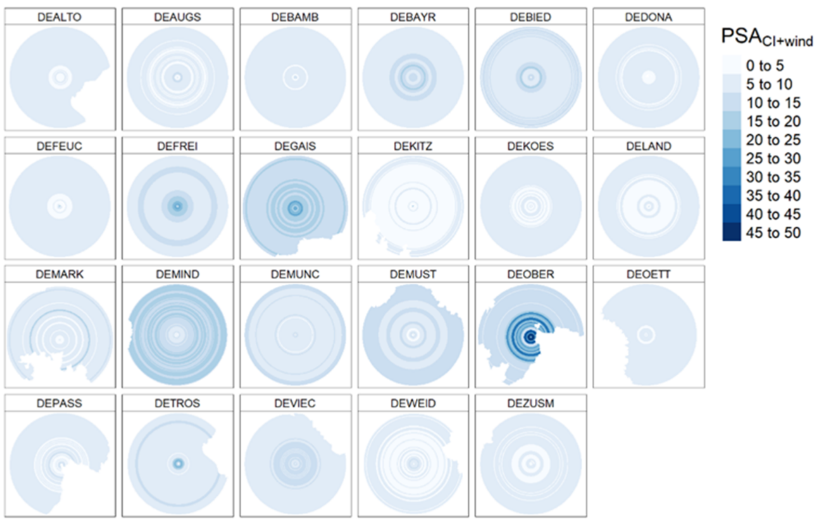

Figure 3. Polar plots of potential source areas weighted by cultivation intensity and wind ($PSA_{CI+wind}$) for 500 m rings within 30 km radius circles around 23 selected pollen stations in Bavaria, Germany. Rings were calculated based on available data within the boundary of Bavaria, Germany. See methods for weighting procedure.

We further subdivided the 30 km surrounding areas of the pollen stations into the close (0–10 km), intermediate (10–20 km), and long (20–30 km) ranges to better characterize the potential source areas. The grassland-dominated stations that showed similar values for close, intermediate, and long distances were DEOBER (close: 28.60, intermediate: 18.73, long: 11.38), DEGAIS (19.08, 14.68, 12.92), and DEMIND (9.09, 15.07, 15.97), where DEFREI exhibited a higher coverage (16.55) only in the close range. In contrast, the pollen station exhibiting the lowest $PSA_{CI+wind}$ was DEKITZ (4.60, 4.34, 4.98).

The potential source area with ($PSA_{CI+wind}$) and without wind weighting (PSA_{CI}) are largely similar, with the overall mean PSA_{CI} of 8.7. Noticeable discrepancies could only be observed for a few stations, where more integrated potential source areas appeared in the inner rings rather than being evenly distributed (see DEOBER or DEMIND for examples in Supplementary Figure S1).

3.3. Concentric Ring Correlations

Pearson correlation coefficients were calculated between the pollen indices (including API and PP) and concentric ring-wise $PSA_{CI+wind}$ across all 23 selected pollen stations (see Figure 4). The correlation coefficients of API and PP with $PSA_{CI+wind}$ varied with distance from the pollen trap. Based on the smoothed trend lines, positive correlation coefficients were derived for distances of up to 10 km. A slightly higher correlation peak of 0.36 was found for API at 2.5 km, while PP resulted in its highest coefficient of 0.33 at the same distance, indicating the predominant impact of sources at close range on the pollen concentrations. Interestingly, for both pollen indices, the innermost 500 m ring was characterized by very low correlation coefficients (API: 0.03, PP: −0.07), which then improved until their maxima were reached at ~2.5 km. For the intermediate and long distances, both correlation coefficients for API and PP increased beyond 10 km again; however, the absolute levels fluctuated around 0. When the pollen indices were correlated with the potential source areas weighted only by cultivation intensity (PSA_{CI}), positive coefficients could be found at the same close distances of less than 10 km (see Supplementary Figure S2). Furthermore, more negative coefficients were found for the outer 30 km. To assess the impact of CI on the results, the CI + 1 and CI^2 + 1 options were applied in addition to the CI^3 + 1 weighting already presented. It was found that PSA weighted by CI^2 + 1 and CI^3 + 1 resulted in only a small change in the correlation coefficients (e.g., 0.4 ± 0.03 in peak at 2.5 km) compared with unweighted PSA (CI + 1).

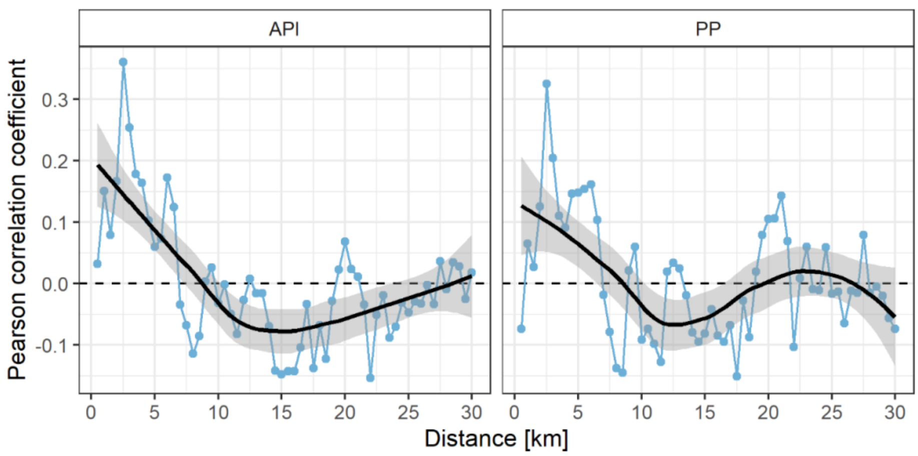

Figure 4. Correlation coefficients between potential source areas and annual pollen integral (API) and pollen peak (PP), respectively, for 23 selected pollen stations in Bavaria, Germany. The potential source areas have been weighted by cultivation intensity and wind ($PSA_{CI+wind}$) for each of the 60 rings. Stations DEUFS/DEGARM/DEBERC/DEHOF were excluded. Loess smoothing was performed using geom_smooth with a span of 0.7.

3.4. Impact of Ring Size and Weighting Technique on the Variability in Grassland Percentages

The applied ring method used rings of equal width (500 m) but different areas, and the grassland percentage was calculated for each ring separately. Based on an analysis showing the variability of the grassland percentage combined for all 23 stations (Figure 5), it can be seen that the variability decreases with distance as the area of the ring increases. The CI weighted grassland percentages in the rings from 0 to 5 km exhibit a high variability with a standard deviation of 7.89. Farther away, the data variability decreases and later stabilizes in longer distances (SD = 3.85).

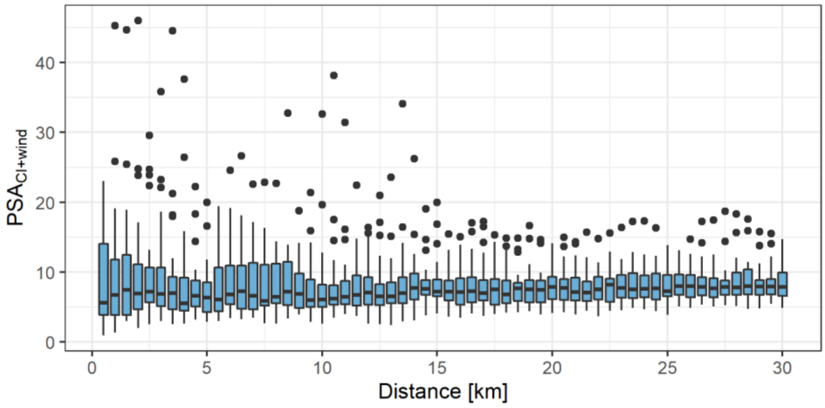

Figure 5. Boxplots showing the variability of potential source areas weighted by cultivation intensity and wind ($PSA_{CI+wind}$) from 23 selected pollen stations per 500 m distance. The corresponding boxplot for PSA_{CI} can be found in the Supplement Figure S3.

The sensitivity analysis revealed that weighting by API levels instead of by land use-specific CI led to an overall increase in the correlation coefficient by 0.11. Apart from the first 500 m, which are highly biased by variability in grassland cover and sampling height (see Section 4.2), the highest increase was observed at a distance of 25.5 km and the lowest at a distance of 1.5 km (Figure 6).

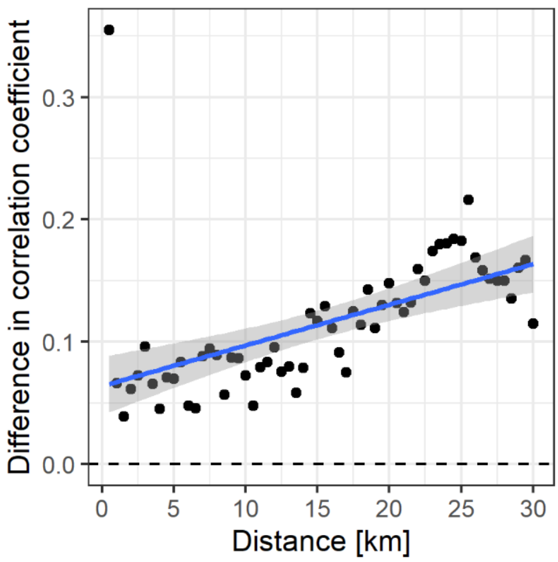

Figure 6. Differences in Pearson correlation coefficients between annual pollen integrals (API) and potential source areas weighted by cultivation intensity and wind ($PSA_{CI+wind}$) before and after manual assignment of API-based CI weightings on non-agricultural areas (OpenStreetMap), where agricultural areas (InVeKos) remained unchanged. Positive values indicate improvements in correlation coefficients after sensitivity weighting.

4. Discussion

Grass pollen loads in terms of annual grass pollen sums (API) and peak values (PP) may be related to the amount of surrounding grassland and management thereof, as well as weather, flowering, emissions and transport, and climate, thus influencing land-use patterns. Based on the values from 27 stations in 2015, API is closely related to the PP ($p < 0.0001$, adj. R2 82.6%), while only two alpine stations (DEBERC and DEGARM) had lower APIs than suggested by the relationship, probably due to a shorter growing season (27 and 36 days, respectively). Grassland percentages in Bavaria depend strongly on the mesoclimate, which can lead to local and regional differences in grassland distribution. The aim of this study was to evaluate the impact of potential wild grass pollen source areas on the pollen concentration in terms of PP and API, considering the influence of land use and management. One major outcome of our analysis is that grassland is not a single homogeneous land-use type, but may comprise at least 33 agricultural (InVeKos) categories, among which meadows and mowing pastures dominate. Meadows and mowing pastures are known for intensive cutting regimes, naturally lowering their strength as pollen sources. A correlation analysis between pollen concentration indices (API and PP) and potential pollen source areas $PSA_{CI+wind}$ showed that pollen counts corresponded more to closer grasslands.

4.1. High Variability in Grass Pollen Concentrations

The Pearson correlation coefficients between annual pollen amounts and potential pollen sources in concentric rings calculated in this study are lower compared to those from a related study on olive pollen [50], which revealed correlation coefficients close to 1 within 50 km. The lower correlation coefficients in the present study can be explained by, in addition to the influencing factors mentioned below, the high number of different grass species contributing to grass pollen load compared to olive. The generally high complexity of grasslands leads to high variability of the measured grass pollen concentrations between pollen stations. Compared to other plant taxa, the *Poaceae* family consists of a huge number of different species worldwide (~9000) [57], with the timing of flowering and the amount of pollen produced varying widely. It has already been shown that the amount of pollen produced per culm can vary six fold depending on species and variety [58]. The vast majority of grasslands are assigned to agricultural holdings (InVeKos database), whereas non-agricultural grassland areas (OpenStreetMap) without the CI weighting PSA_{CI} account for only a small share of ~10%. Among the agriculturally cultivated grasses, arable grasslands with cereals have a special position, although they belong to the *Poaceae* family. From the cereal species, only rye (*Secale cereale*) and maize (*Zea mays*) are anemophilous (wind-pollinating) [39], while all other cereal species are autogamous (self-pollinating). Based on the InVeKos database, rye constitutes a very small total share of ~0.55% (39.000 hectares) in Bavaria, while maize is cultivated with a total share of ~7.80% (550.000 ha). In general, cereal pollen is characterized by heavy weight and large size, and maize pollen additionally has a sticky surface. Those properties make them unlikely to be transported over distances of more than 500 m [39,59]. Related experiments determined that the pollen concentration decreases greatly after 100 m [60], e.g., after 20 m, the concentrations would be less than 0.1% [61]. Therefore, the impact of cereal pollen is highly limited to the immediate surroundings of the emission source.

Differences in the species composition based, e.g., on altitude or soil-climate zone can be seen in grasslands systems [18,19]. The variety of grasses from agricultural use is expanded by a high number of adapted cultivars for Germany [62]. On both agricultural and non-agricultural grasslands, the cultivation intensity covers a wide spectrum from uncut to high cutting frequencies (up to seven times per year), where the most decisive factor is the timing of first cut; this contributes to the complexity of analyzing the grasslands. On the microscale, grasses are abundant on various types of small patches, which are supposed to be mostly unmanaged grassland. From the available data, those small grass patches cannot be fully identified, despite having a considerable impact on the overall pollen concentration. For the present study, we also assume that for most southern stations, which are characterized by a higher grassland percentage, pollen emission and local transport might be hampered by more frequent rainy days.

4.2. Low Influence of Grass Pollen Source Areas

We had expected that the correlation coefficients, regardless of their weighting, would reach their maximum closest to the pollen stations, thus mainly reflect the grassland near the pollen trap. However, as they are strongly affected by the high variability of the potential source areas of grasslands, especially in the first rings and their smaller areas, the respective correlation coefficients are lower than expected. Additionally, the low correlation coefficients in the first rings can probably be explained by the geographical location of the pollen traps in settlements and the height difference between the ground level and the installation height of the pollen trap. According to international standards, pollen traps should be installed 10 to 20 m a.g.l. [63], and suitable installation sites are usually found within cities at public institutions [20].

The installation height of pollen traps is critical for wind-pollinating plant species, as the pollen transport range could differ from meters to hundreds of kilometers. Owing to the large aerodynamic diameters (35 µm aerodynamic and 45 µm geometric) and high pollen weight (~20 ng) of common wild grass pollen [64], grass pollen transport is largely limited to local and regional scales (up to 100 km). Therefore, the installation height of the pollen traps is not specifically suitable for grass pollen. In this context, the complex transportation dynamics from the ground level where pollen is emitted up to 15 m a.g.l. is expected to strongly influence the correlation coefficients in the closest surroundings as well [65].

4.3. Potential Impacts of Ring Size and Weighting Techniques

When applying the CRM, ring sizes continuously increase in area with distance, which means that the variability of potential source areas weighted by cultivation intensity and wind declines with the distance. Since the spatial variability in the API and PP among the 23 pollen trap sites is quite high, this may have affected the correlation coefficient.

In the ring method, the grass pollen sources of the individual rings are correlated with the respective pollen concentrations, whereby a distance weighting is achieved via the largely differing absolute ring size. Thus, the distance of the relevant source regions can be estimated by this method. An alternative, if the decline with increasing distance is known, could be to include distance weighting, as used in paleontological research to study past land cover, where the influence of distance is taken into account from the dispersal models at the beginning and only one value is calculated for the potential pollen sources for each site [66,67]. Weighting the PSA with CI and wind showed little effect on the correlation coefficients. A possible explanation for the small effect of CI could be the different percentages of grassland at each intensity level (see Table 2). The improvement in the correlation coefficients due to wind weighting might be stronger if the wind direction and wind speed were recorded on site at the pollen traps, which would certainly facilitate the detection of potential pollen emission areas. The separation of the concentric rings into four quarters (i.e., wind directions NE, SE, SW, and NW) still might have been too wide, as the variability of the weighted grassland index among the quarters in the outer ring is low.

Agricultural grasslands mainly comprise meadows (InVeKos 451) and mowing pastures (InVeKos 452), which are moderately to intensively cultivated. In this context, the applied weighting is necessary to emphasize the importance of smaller, less intensively managed patches, such as field margins (InVeKos 68) or Natura 2000 conservation areas (InVeKos 958).

The sensitivity analysis (see Figure 6) revealed that the API-based weighting of the non-overlap OpenStreetMap (i.e., non-agricultural sources) enhanced the correlation coefficients, especially in the medium to long range. The enhancement of the correlation coefficients by the ideal case of API weighting in the distances from 0 to 10 km led to a small increase in the correlation coefficients by 0.04 to 0.10, except in the first 500 m. Such patterns indicate that the $PSA_{CI+wind}$ results in a high correspondence with the measured pollen indicators for close distances, but does not represent the measured pollen concentration at the medium to long range well.

5. Conclusions

It can be concluded that the applied CI and wind weighting method is suitable, especially for the investigation of local pollen sources and their contribution to the measured wild grass pollen concentration. This is the first study combining high-resolution land-use data weighted by cultivation intensity and wind direction with wild grass pollen concentrations. We demonstrate that the weighted land-use data from InVeKos and OpenStreetMap can explain, to certain extent, the pollen captured in the pollen stations. Still, there is a considerable amount of variability that we cannot attribute to the grassland percentage within the vicinity of the pollen traps. This variability can instead be explained by small-scale conditions in the immediate surroundings and long-distance transport at larger distances. Subsequent studies should focus on mapping the potential pollen sources combined with a probability index of flowering or pollen shedding. This may help improve the situation for those allergic to grass pollen by suggesting specific measures to manage pollen-emitting grasslands.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/land11020306/s1, Figure S1. Polar plots of potential source areas weighted by cultivation intensity (PSA_{CI}) for 500 m rings within 30 km radius circles around 23 selected pollen stations in Bavaria, Germany. Rings were calculated based on available data within the boundary of Bavaria, Germany. See methods for weighting procedure; Figure S2. Correlation coefficients between potential source areas and annual pollen integral (API) and pollen peak (PP), respectively, for 23 selected pollen stations in Bavaria, Germany. The potential source areas have been weighted by cultivation intensity (PSA_{CI}) for each of the 60 rings. Stations DEUFS/DEGARM/DEBERC/DEHOF were excluded. Loess smoothing was performed using geom_smooth with a span of 0.7; Figure S3. Boxplots showing the variability of potential source areas weighted by cultivation intensity (PSACI) from 23 selected pollen stations per 500 m distance.

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ORIGINAL PAPER

Establishing the twig method for investigations on pollen characteristics of allergenic tree species

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Abstract
The twig method in climate chambers has been shown to successfully work as a proxy for outdoor manipulations in various experimental setups. This study was conducted to further establish this method for the investigation of allergenic pollen from tree species (hazel, alder, and birch). Direct comparison under outdoor conditions revealed that the cut twigs compared to donor trees were similar in the timing of flowering and the amount of pollen produced. Cut twigs were able to flower in climate chambers and produced a sufficient amount of pollen for subsequent laboratory analysis. The addition of different plant or tissue fertilizers in the irrigation of the twigs did not have any influence; rather, the regular exchange of water and the usage of fungicide were sufficient for reaching the stage of flowering. In the experimental setup, the twigs were cut in different intervals before the actual flowering and were put under warming conditions in the climate chamber. An impact of warming on the timing of flowering/pollen characteristics could be seen for the investigated species. Therefore, the twig method is well applicable for experimental settings in pollen research simulating, e.g., accelerated warming under climate change.

Keywords Pollen · Allergy · Climate change · Twig experiment

Introduction

Under climate change, the relevance of aeroallergens including allergenic pollen is expected to increase (Beggs 2004). Nowadays, pollen-related allergic rhinitis already prevails in up to 40% of the population in Europe (D'Amato et al. 2007). New techniques for the investigation of allergenic pollen are steadily being developed (Sofiev and Bergmann 2013).

The goal of these studies was among others to identify key influences on seasonal patterns and pollen emission, including the effects of climate change (Marselle et al. 2019), and to study pollen characteristics, such as variation in allergic content among different grass species (Jung et al. 2018). It is already known that climate change alters seasonal patterns of pollen, e.g., leading to an earlier start of pollen season (Frei and Gassner 2008; Menzel et al. 2006; Menzel et al. 2020a, b; Rosenzweig et al. 2008). Seasonal patterns and their trends are normally captured by phenological studies and/or longer time series of measured airborne pollen concentrations (e.g., Menzel et al. 2021). In general, pollen characteristics, such as protein content, and allergenicity are obtained from airborne samples using high-volume impactors (Buters et al. 2010; Grewling et al. 2020) or low-volume devices such as multi-vial cyclone samplers (Brennan et al. 2019). However, the acquisition costs for such devices are high and even then, it cannot be ruled out that pollen from more than one individual is collected. Therefore, the study of pollen characteristics of individual species such as birch or hazel usually requires in situ collection of pollen, which should ideally be combined with manipulative experiments on climate change. For the subsequent laboratory analysis, well-established standard methods such as ELISA and Western blot for the determination of allergenic content are used (Bufe et al. 1996; Jung et al. 2018; Schäppi et al. 1999). The necessary in situ collection of pollen is often associated with difficulties, as the pollen has to be stored under suitable conditions immediately after emission. Therefore, pollen collection requires, e.g., air-

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permeable bags that collect the pollen while protecting it from rain and insects and at the same time likely formation of condensed water has to be prevented. Glassine bags which are commonly used in plant crossing attempts offer air permeability but do not fulfill the other requirements (Hanna 1990; Jung et al. 2018).

Here, the twig method in climate chambers may also meet these requirements for in situ pollen collection allowing at the same time variations in environmental conditions. Thus, this method has the benefit that in the face of climate change different climatic scenarios and their impacts on pollen characteristics can be studied. The twig method has already been well described in previous studies (Basler and Körner 2012; Dantec et al. 2014; Menzel et al. 2020a, b; Primack et al. 2015). Vitasse and Basler (2014) observed for the tree species hornbeam, European beech, and sycamore that the timing of budburst is comparable between cut and uncut twigs from a donor tree. Among others, the twig method can be applied for various phenological observations, e.g., to test the influence of winterly (chilling) temperatures (Laube et al. 2014a) and bud development under elevated air humidity (Laube et al. 2014b).

From the literature, it is known that under warmer spring temperatures, flowering is generally accelerated (Fu et al. 2012; Wang et al. 2020). In the study of Fu et al. (2012), potted saplings from beech, birch, and oak were treated with various temperature manipulations in climate chambers. It revealed that spring warming is more influential on budburst than winter warming and that spring warming (forcing) leads to an earlier budburst. A strong correlation between temperatures in March and the start of pollen season/in situ observed flowering phenology was also observed for birch (Bogawski et al. 2019; Newnham et al. 2013). Another study on birch (Miller-Rushing and Primack 2008) reported that the inflorescence length does not influence the start of flowering. Other studies have shown that the responses to changed climatic conditions are highly species-specific (Laube et al. 2014a; Malyshev 2020; Miller-Rushing and Primack 2008).

The objective of this study was to establish the twig method in climate chambers for the investigation of allergenic pollen. In order to observe the impact of harvesting on flowering and pollen characteristics, an outdoor comparison between cut and uncut inflorescences regarding their pollen production and pollen characteristics was conducted. We examined whether branch experiments in climate chambers can be used as a proxy for outdoor manipulations on pollen. In pollen research, the harvesting of twigs shortly before flowering is required, and then the pollen has to be emitted under controlled conditions in the climate chamber. Furthermore, it was evaluated under controlled conditions in the climate chamber whether the usage of different fertilizers as an additive in the irrigation causes favorable/unfavorable conditions for pollen production and has an impact on pollen characteristics as previous studies suggested for flowering and number of inflorescences (AL-Kahtani and Ahmed 2012; Maksoud 2000).

Material and methods

Investigated species and tree selection

We chose the widespread shrub species *Corylus avellana* (hazel) and tree species *Alnus glutinosa* (common alder) and *Betula pendula* (silver birch), which are characterized by a high allergenicity of their pollen and the availability of common allergens (cross-reactive Bet v1) (Biedermann et al. 2019; Niederberger et al. 1998; Weber 2003). These species are monoecious with typical long catkins containing only male flowers (Filbrandt-Czaja and Adamska 2018). Hazel and alder flower early in January to March, whereas birch flowers later in March to June (only in northern latitudes).

The sampling took place on the specimen in the 3 km surroundings of the campus of the Technical University of Munich at Freising/Weihenstephan (48.400292 N, 11.716874 E). Care was taken to ensure that the selected trees had a minimum distance of 15 m from buildings and varied in age, diameter, and height. From this selection, 3 hazel, 2 alder, and 2 birch tree specimen were chosen. Heights were measured with a relascope, diameters were measured with a tape measure, and ages were estimated. The selected hazels were approximately of the same age (20 years old), height (8 m), crown length (6 m), and diameter (20 cm) and had similar densities to male catkins. The two alder trees were likely of the same age (25 years old), height (15 m), crown length (13 m), and diameter (30 cm) and had about the same number of male catkins. The two birch specimens were of different estimated age (30 and 50 years old), height (15 and 25 m), crown length (13 and 23 m), and diameter (30 and 40 cm).

Harvest and treatments of twigs

The investigation period was from the 5th of November 2018 to the 18th of April 2019. Twigs of the respective species and specimens were harvested in weekly intervals before the actual flowering (Table 1). It was assured that the harvested twigs (length 40 to 50 cm) had the required minimum number of five male inflorescences. The sampling height was 2–3 m above the ground level. After cutting, the twigs were disinfected with 90% ethanol and their basis was recut underwater to avoid disruption of water uptake. The twigs were then stored in glass bottles (capacity 100 ml) which were filled with tap water.

Previous experiments suggested that the usage of different fertilizers as an additive in irrigation may have influence on development of the inflorescences (AL-Kahtani and Ahmed 2012; Maksoud 2000). Therefore, hazel was treated

Table 1 Overview on harvested twigs including the following: species, specimen number, flowering location (indoors or outdoors); cutting date; number of twigs analyzed in the laboratory (number of twigs exclusively analyzed for allergen content in square brackets []): indoors: climate chamber (CC) and window sill (WS) subdivided into treatments control (C), plant fertilizer (PF), tissue fertilizer (TF), and outdoors: into the treatments cut and uncut; Flowering date refers to the observation date where BBCH 60 was recorded

Species	Specimen	In-/ outdoors	Cutting date	Number of twigs analyzed in the laboratory	Flowering [BBCH 60]
Hazel	1 and 2	Indoors	12-06-2018	CC (5 [2] C, 4 [1] PF, 8 [2] TF), WS (5 [2] C, 3 [1] PF, 4 [2] TF)	12-13-2018
Hazel	1 and 2	Indoors	12-12-2018	CC (4 [2] C, 3 [1] PF, 7 [1] TF), WS (5 C, 4 [3] PF, 2 TF)	12-18-2018
Hazel	3	Outdoors	02-13-2019	3 [1] cut, 5 [1] uncut	02-19-2019
Alder	1 and 2	Indoors	01-21-2019	CC (1 [1] C, 4 [1] TF), WS (3 [2] C, 2 [1] TF)	01-31-2019
Alder	1 and 2	Outdoors	02-13-2019	2 [1] cut, 3 [2] uncut	03-06-2019
Alder	1 and 2	Outdoors	02-25-2019	3 [2] cut, 2 [1] uncut	03-06-2019
Alder	1 and 2	Outdoors	02-28-2019	1 [1] uncut	03-06-2019
Birch	1 and 2	Indoors	03-12-2019	CC (6 [2] C, 4 [2] TF), WS (4 C, 3 [1] TF)	03-21-2019
Birch	2	Indoors	03-29-2019	CC (3 [1] C, 3 [2] TF), WS (3 C, 2 TF)	04-04-2019
Birch	2	Indoors	04-04-2019	CC (5 [1] C), WS (5 [1] C)	04-04-2019
Birch	2	Outdoors	03-22-2019	1 [1] uncut	04-04-2019
Birch	2	Outdoors	03-23-2019	1 [1] uncut	04-04-2019
Birch	2	Outdoors	03-29-2019	2 cut, 3 [1] uncut	04-04-2019
Birch	2	Outdoors	04-04-2019	4 [2] cut, 7 [3] uncut	04-04-2019
Birch	2	Outdoors	04-06-2019	4 [1] cut, 5 [1] uncut	04-04-2019
Birch	2	Outdoors	04-09-2019	6 [1] uncut	04-04-2019

respectively with pure water, tissue fertilizer (B5 medium—100 ml/liter), and plant fertilizer (nitrogen (N) 8%, phosphorus (P_2O_5) 8%, potassium (K_2O) 6%—1 ml/liter) (Table 1). Since the first sampling series on hazel twigs did not show any difference in the development of inflorescences between the fertilizers, twigs of alder and birch were treated with pure water and tissue fertilizer only. Additionally, to each substrate, Chrysal Clear was added as remedy against mold growth.

The twig samples were then stored in the climate chamber which was running under a day-night cycle of 15 and 9 h, with 20 °C and 15 °C air temperature, respectively (Fig. 1). As backup in case of technical failures, a second set of samples was kept under room conditions on a window sill within the university building. Those conditions did not reflect any climate scenario; they were merely used to achieve flowering and pollen shedding.

Comparison of cut and uncut twigs on donor trees

For hazel, alder, and birch, on-tree comparisons between cut and uncut twigs under outdoor conditions were conducted. Single twigs with a minimum of five inflorescences were cut from the donor trees at different time intervals before natural flowering (Table 1) and directly put into plastic water containers (capacity 25 ml) which were afterwards attached vertically with wires to their original position. As a control, on the respective cutting dates, the same number of uncut twigs was selected on the donor trees in the surrounding of the cut twigs. Shortly before flowering, the cut and the selected uncut branches were covered with glassine bags for the in situ collection of pollen. After the pollen emission was completed, the twigs with their glassine bags were cut.

Fig. 1 Pollen collection in glassine bags from left to right: window sill, climate chamber, outdoor conditions

Before separating the pollen from the anthers, the glassine bags had to be dried for approximately 1 week. Since the bags were only made of a material similar to paper, the outdoor in situ pollen collection was heavily impacted by precipitation (rain and snow) as well as by condensed water. The pollen already emitted into the bags got affected by humidity when the bags were soaked through. The inherent problem was that pollen partly burst into small pieces and after drying could not be separated from the paper anymore. To overcome this issue, we tried to cover the glassine bags additionally with plastic bags filled with a desiccant. Unfortunately, after a short time, condensation water had accumulated in the plastic bags causing similar issues as before. Still, part of the samples could be used for further laboratory analysis.

Phenological observations and climatic parameters

During the study period from the 5th of November, 2018, to the 18th of April, 2019, we recorded phenological stages once or twice a week according to the BBCH code (Meier 2001), with a particular focus on the period with sporadically first flowers open (BBCH 60). The BBCH was recorded indoors (climate chamber and window sill) and outdoors on the sampling trees. Temperature and air humidity were continuously measured in 30 min temporal resolution by Hobo Loggers (type U23 Pro v2), always at the height of the samples.

Laboratory analysis

After completion of flowering, the bags were inverted and gently shaken, resulting in accumulation of pollen at the bottom of the bags. After removal of the branches, the catkins/anthers were manually separated from the pollen using tweezers. The remaining pure pollen was finally weighed. For each twig, the average pollen amount per catkin and the average catkin length were determined. The weighted pollen was stored at −20 °C.

The subsequent laboratory analysis comprised the measurement of mean pollen weight per grain, protein content per grain, and allergen content. 5 mg pollen was weighed in, and then 1 ml buffer was added, and 20 μl of this mixture was given onto a slide which was then inserted into a cell counter (Bio-Rad; type TC10) for automatic counting. The average pollen grain weight was derived from the initial weight (5 mg) and the count from the instrument.

Before protein and allergen quantification, the protein had to be extracted from the pollen. For this purpose, 10 mg pollen given in 1 ml buffer was shaken continuously for 3 h. The supernatant was stored at −20 °C. For protein quantification, we tested the BCA (Pierce BCA™ Protein Assay) and Bradford (Bio-Rad Protein Assay Dye Reagent Concentrate) methods in a pre-test with purification of the extract and gel electrophorese. The results of the Bradford test for protein content were in good agreement with other related studies (Ozler et al. 2009; Schäppi et al. 1997), while the results obtained with the BCA method showed much higher values. Therefore, protein quantification as protein content per pollen grain was performed using the Bradford test only. To determine allergen content, the Western blot technique was applied, which uses three different antibodies: human antibody (sera mixture of 29 patients who are allergic to Birch), monoclonal anti-human IgE antibody produced in mouse (Sigma-Aldrich), and rat anti-mouse IgG2b (–HRP conjugated, produced by The Monoclonal Antibody Core Facility at the Helmholtz Center). After the application of all three antibodies, the allergens were detected in the last step under chemiluminescence (ECL™ select Western blotting detection reagent) (Fig. 2). Among the allergens visualized, the allergen Bet v1 showed the clearest signal as it is highly abundant. Bet v1 is cross-reactive in hazel, alder, and birch and can therefore be detected in all three species. For the calculation of Bet v1 allergen content, the signal intensity of chemiluminescence was extracted using the Fiji package from ImageJ (Rueden et al. 2017; Schindelin et al. 2012) and the protein content was determined via Ponceau S staining during Western blotting. The allergen content was then calculated by dividing signal intensity and protein content, thus it is a relative value without unit. All laboratory analyses were conducted in cooperation with the Helmholtz Center.

Statistics

The statistical analysis was conducted in RStudio (version 3.5.1/2018-07-02). The per treatment distributions of the parameters average catkin length (cm), average pollen weight per catkin (mg), pollen weight per grain (ng), protein content per grain (ng), and allergen content (unitless) were tested by the Shapiro-Wilk test for normality. Since the parameters were not normally distributed (p-value of Shapiro-Wilk test < 0.05), non-parametric tests were further used: Student's t-test for comparisons between two groups, the univariate ANOVA (Kruskal-

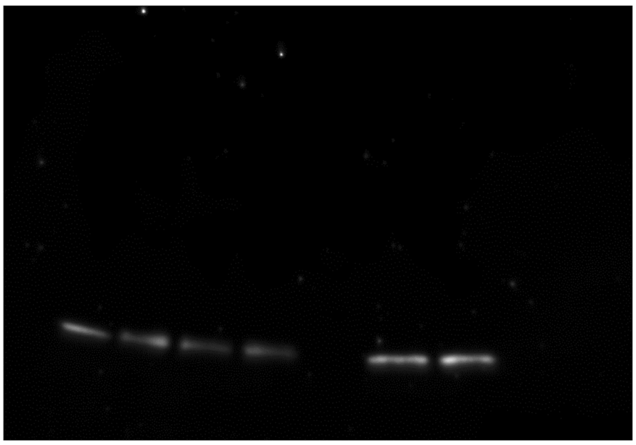

Fig. 2 Western blot—chemiluminescence measurement for six alder samples, measurement time 100 s

Wallis test) for comparisons with more than two groups, and subsequently using pair-wise Wilcoxon tests for individual group comparison. p-values less than 0.05 were considered statistically significant. Due to the limited number of samples per treatment which could be analyzed for the pollen weight per grain (ng) and allergen content, no statistical test could be performed and thus, only differences are shown in the results.

Results

During the investigation period, temperature and relative air humidity outdoors were on average 3.7 °C and 81.9% respectively, whereby the minimum daily air temperature of −6.2 °C was reached on the 20th of January 2019 and the maximum air humidity of 99.9% on the 11th of January 2019, and the maximum of 23.6 °C and the minimum relative air humidity of 26.7% both on the 18th of April 2019. Indoors, in the climate chamber, 16.8 °C and 73.3% and on the window sill 18.6 °C and 35.3% (air temperature and humidity respectively) were recorded on average (Fig. 3).

Hazel was harvested for the indoor experiments twice on the 6th and 12th of December 2018, for the outdoor cut/uncut investigation twigs were cut/selected on the 13th of February 2019. First flowering (BBCH 60) both in the climate chamber/ on the window sill was observed for the twigs cut on the 6th of December 2018 on the 13th of December 2018 and for the twigs cut on the 12th of December on the 18th of December, under outdoor conditions for both the cut and selected twigs on the 19th of February 2019 (Table 1).

Between the two fertilizers and the control, significant differences were found in protein content ($p<0.057$), and no significant differences in length per catkin ($p=0.28$) and in pollen weight per catkin ($p=0.11$) (Fig. 4). There were no significant differences in any of these parameters between the two harvesting dates (the 6th and 12th of December 2018). In comparison to the outdoor conditions, the catkin length and the weight per pollen grain were significantly smaller in the climate chamber/window sill ($p=0.031$ and $p=0.0013$). Under outdoor conditions, the cut treatment showed a higher average pollen grain weight (14.4 ng) than the uncut treatment (6.5 ng). For the allergen content, an opposite behavior was observed. The uncut treatment had higher allergen content (2.3) than the cut treatment (1.7).

Alder was harvested on the 21st of January 2019 for indoor investigation; outdoor twigs were cut/selected on the 13th, 25th, and 28th of February 2019. Flowering in the climate chamber/window sill was recorded on the 31th of January 2019, under outdoor conditions on the 6th of March 2019. Between the treatments tissue fertilizer and the control, no significant differences were found (Fig. 5). There were significant differences in the protein content between climate chamber/window sill and outdoor conditions ($p=0.046$). For the catkin length and the pollen weight in the climate chamber/window sill, no results were available. Under outdoor conditions, the average pollen grain weight (ng) was higher for the uncut (12.7 ng) than the cut treatment (9.2 ng).

Birch was harvested on the 12th and 29th of March and the 9th of April 2019; outdoor twigs were cut/selected on the 22nd, 23rd, and 29th of March and on the 4th, 6th,

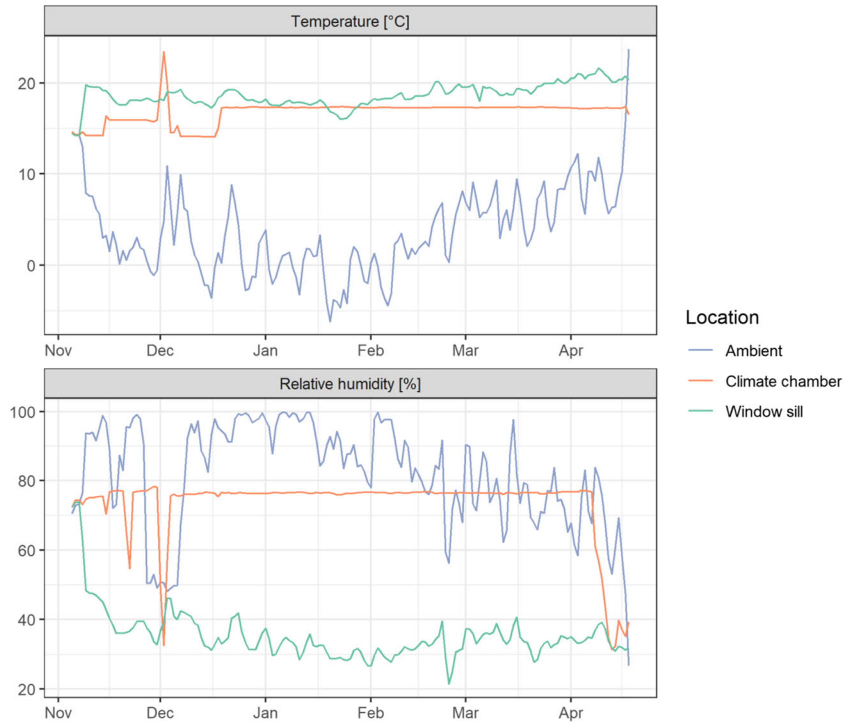

Fig. 3 Temperature (°C) and relative humidity (%) indoors at the window sill, in the climate chamber, and outdoors during the investigation period 5th of November 2018 to 18th of April 2019

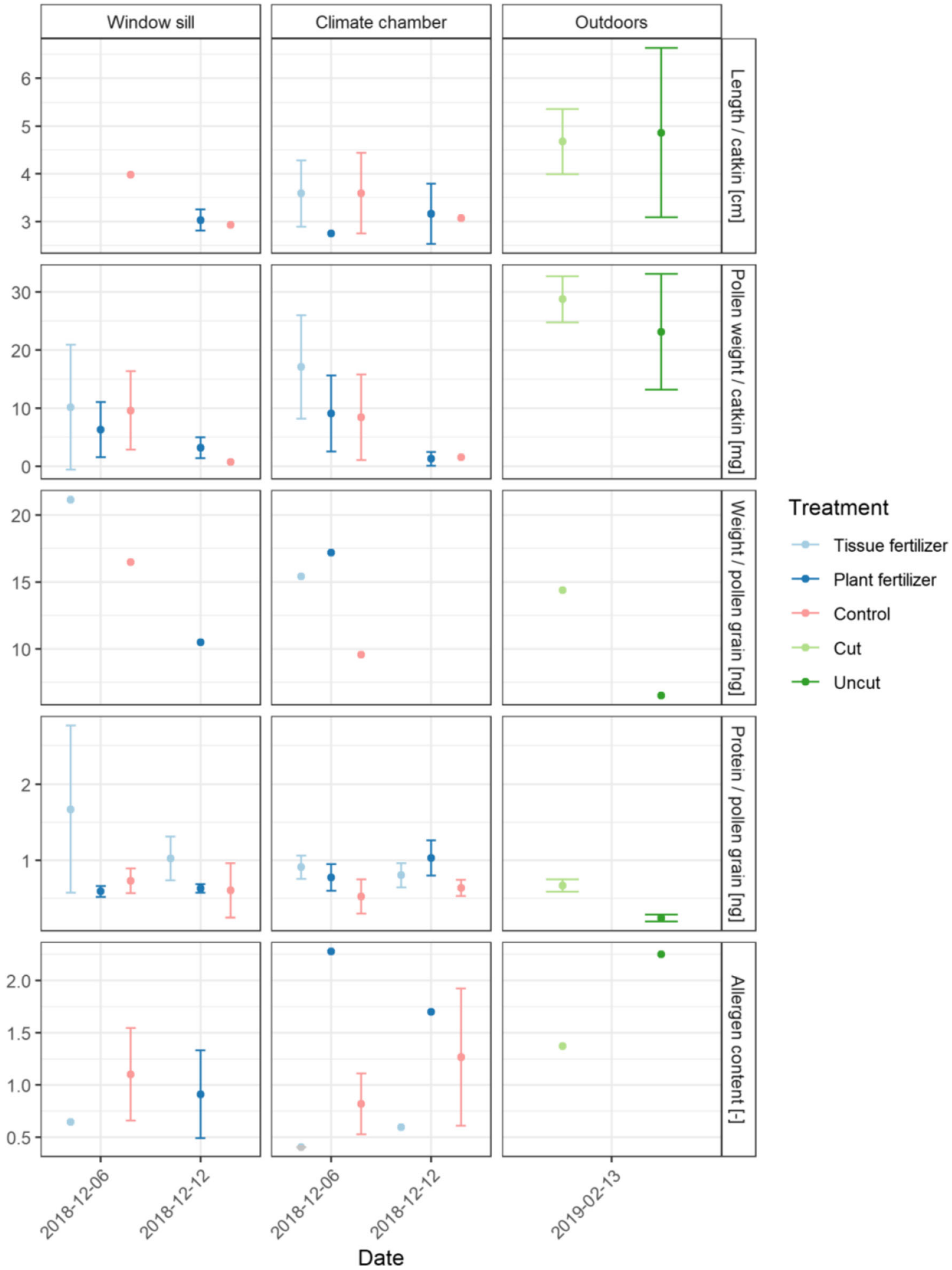

Fig. 4 Results of the laboratory analysis for hazel: length per catkin (cm), average pollen grain weight (ng), protein content per pollen grain (ng), and allergen content (unitless) for the respective indoor flowering locations window sill and climate chamber, as well as outdoors under the treatments tissue fertilizer, plant fertilizer, control, cut and uncut for different harvesting dates; bars represent standard deviation according to the sample size

and 9th of April 2019. Flowering in the climate chamber/ window sill from the twigs cut on the 12th of March was observed on the 21th of March 2019 and for the two later cuttings on the 29th of March and 4th of April flowering could be monitored on the 4th of April. Outdoors flowering was observed on the 4th of April 2019. There

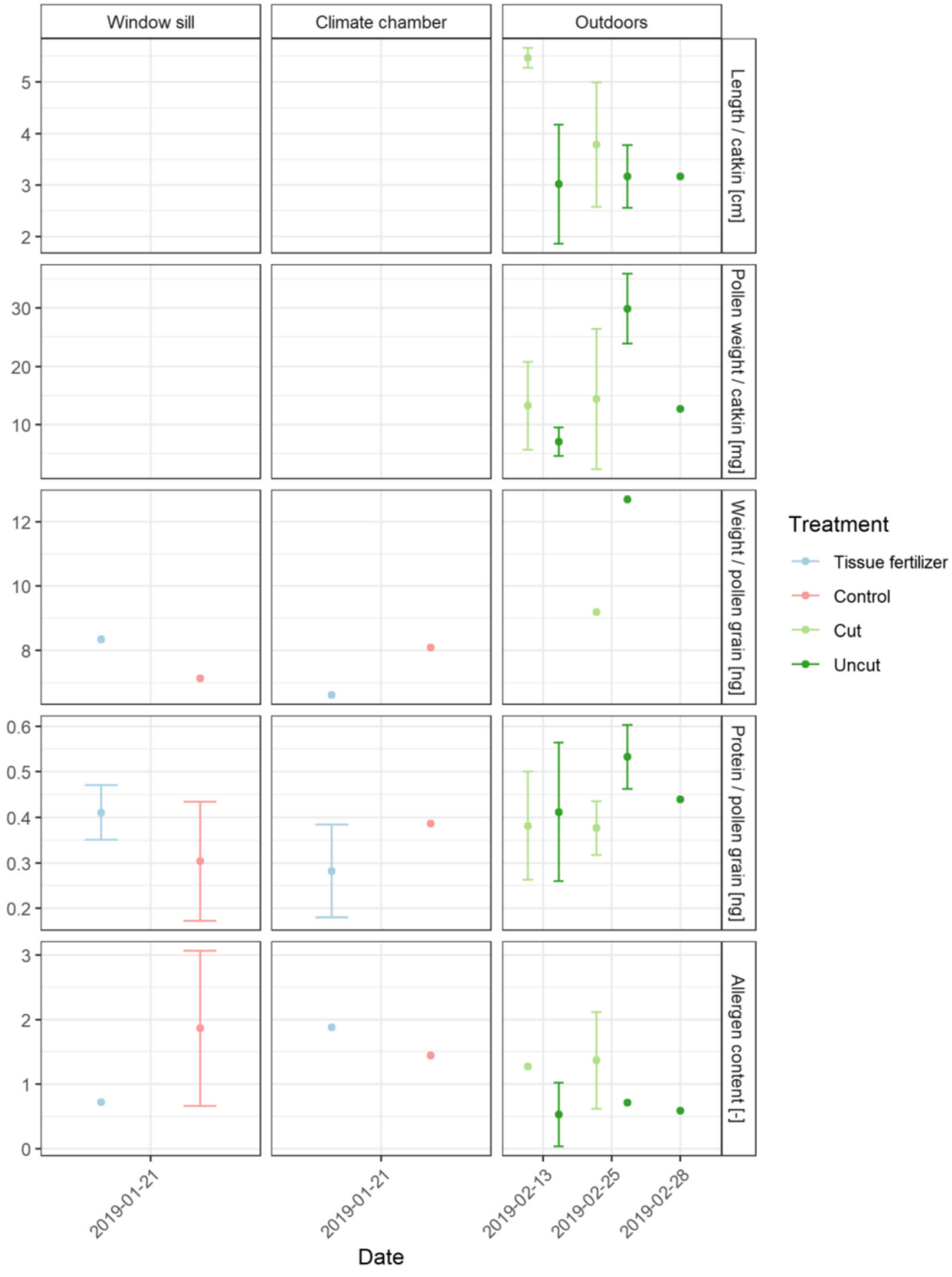

Fig. 5 Results of the laboratory analysis for alder: length per catkin (cm), average pollen grain weight (ng), protein content per pollen grain (ng), and allergen content (unitless) for the respective flowering locations window sill and climate chamber, and outdoors under the treatments tissue fertilizer, plant fertilizer, control, cut and uncut for different harvesting dates; bars represent standard deviation according to the sample size, missing data for length per catkin (cm) and protein content per pollen grain (ng) for the cutting date 21th of January 2019

were no significant differences between the treatments tissue fertilizer and control in catkin length, pollen weight per catkin, and protein content per grain (Fig. 6). Under controlled conditions in the climate chamber/window sill,

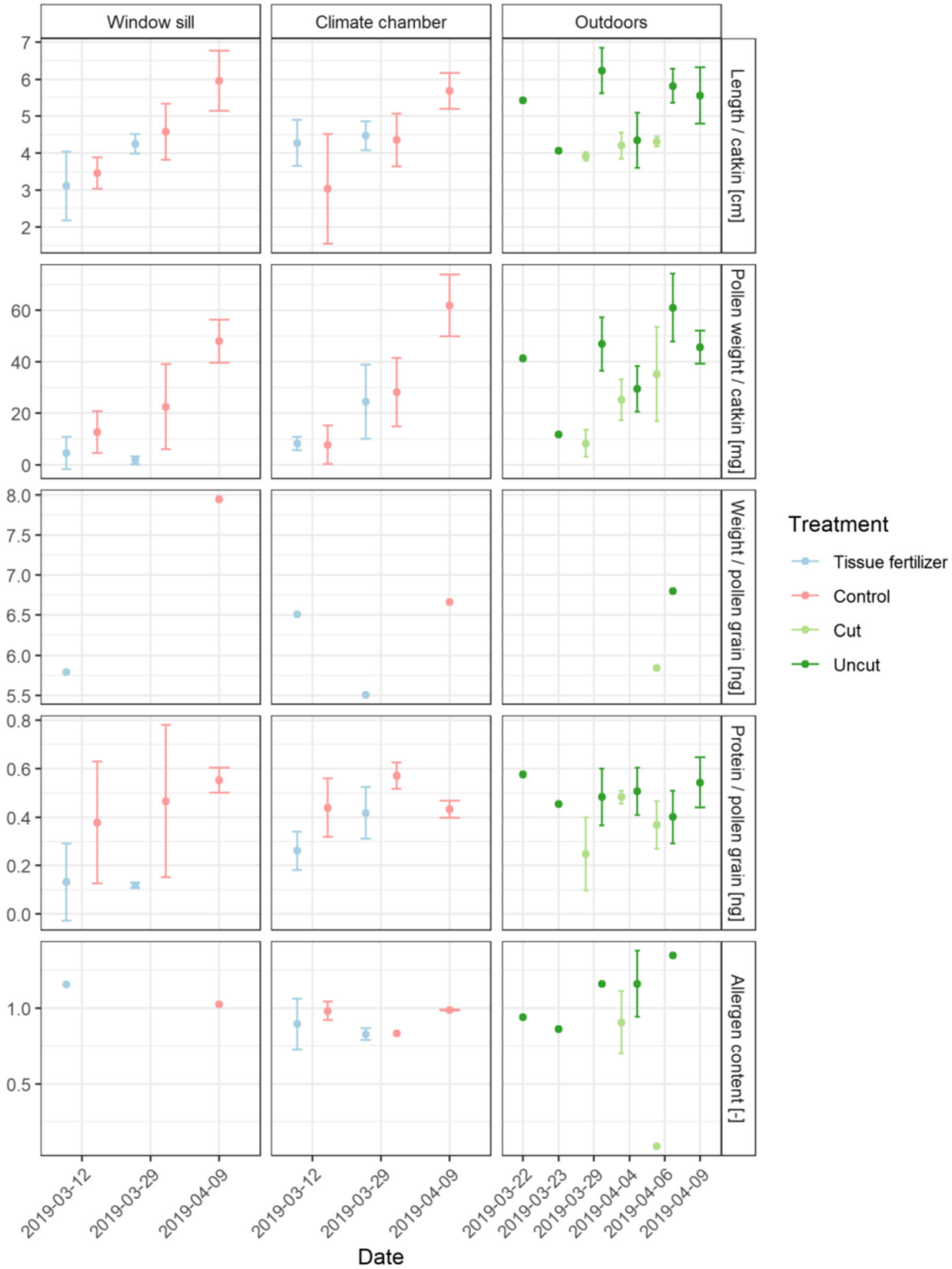

Fig. 6 Results of the laboratory analysis for birch: length per catkin (cm), average pollen grain weight (ng), protein content per pollen grain (ng), and allergen content (unitless) for the respective flowering locations window sill and climate chamber, and outdoor conditions under the treatments tissue fertilizer, plant fertilizer, control, cut and uncut for different harvesting dates; bars represent standard deviation according to the sample size

later, harvesting revealed higher values for the catkin length, the pollen weight per catkin, and the protein content. The results were significant for the parameters catkin length (p=0.0054), pollen weight per catkin (p=0.023) but not for the protein content (p=0.27). For the comparison between outdoor and the climate chamber/window sill, significant differences were found for the catkin length (p=0.031) and pollen weight per catkin (p=0.0064) but not for the protein content (p=0.09). The average pollen grain weight was in the uncut treatment higher (6.8 ng) than in the cut treatment (5.9 ng).

Discussion

The main objective of this study was to establish the twig method in climate chambers for the investigation of flowering phenology and pollen characteristics of allergenic shrub/tree species. In order to evaluate whether the cutting of twigs influenced flowering, pollen emission, and pollen characteristics, a comparison of cut and uncut twigs outdoors on donor trees was conducted. The studied tree species hazel, alder, and birch showed in most cases (except birch on the 29th of March 2019) only marginal differences in the studied parameters (except average pollen grain weight and allergen content) for the outdoor comparison between cut and uncut branches. For allergen content and pollen weight per pollen grain, a higher sample number would be required for a solid assessment. Flowering occurred on the same day, the pollen amount emitted per catkin was similar and differences in the pollen characteristics were minor and not significant. Variations in the results for different twig selecting dates (uncut treatment having the same harvesting date) can be explained by a varying location of the selected twigs on the respective donor tree. Based on the results of the cut and uncut comparison on the donor trees, the usage of cut twigs can be recommended as appropriate for flowering and pollen investigations.

Furthermore, it was studied whether the usage of different fertilizers as an additive in irrigation has an influence on the vitality of the inflorescences and other observed parameters. Dormant twigs cut before natural flowering from hazel, alder, and birch were kept indoors under constant conditions in climate chamber or room condition (window sill) until full flowering and pollen emission. The respectively added plant and tissue fertilizer in contrast to other studies (AL-Kahtani and Ahmed 2012; Maksoud 2000) neither affected plant vitality nor the observed parameters. The weekly exchange of water seemed to be sufficient for proper inflorescence development as potentially occurring mold (Criado et al. 2005) could in the meantime be prevented by the addition of a remedy against fungal growth.

In order to study the possibility of using climate chamber experiments as proxy for outdoor manipulations, twigs were harvested in different intervals (see Table 1) before natural outdoor flowering and were kept in the climate chamber/room conditions for flowering. In comparison to the outdoor conditions (average temperature 3.7 °C), a much earlier flowering was observed in the climate chamber (average temperature 16.8 °C) for hazel (−69 days), alder (−35 days), and birch (−15 days), ordered by their harvesting dates beginning of December, end of January, and beginning of March. Wang et al. (2020) observed similar trends in a climate chamber experiment on branches of six Asian woody species, where a 3 °C increase in spring temperature resulted in flowering advanced by 2.3 to 36.1 days, depending on the species. In the present study, an impact of the flowering time could be observed on the pollen amount produced, and on pollen characteristics for all investigated species. In the climate chamber, later branch harvesting and thus later flowering resulted in higher pollen production per catkin, catkin length, and protein content. This could be seen in the respective results from the climate chamber/window sill as compared to the outdoor conditions. For birch, the same dependency could be seen for the different harvesting dates between the 12th of March and the 9th of April. A study on birch from (Buters et al. 2010) showed a similar dependency for the allergen content, it strongly increased in the days before flowering. Related studies on birch have shown that climate warming can affect leaf size and the number of shoots developed (Hofgaard et al. 2010), as well as leaf phytochemistry (Jamieson et al. 2015).

Since the pollen weight per grain was not investigated for each harvesting date separately (due to lack of pollen amount), further experiments would be needed. The generally considerably lower relative humidity on the window sill (room temperature) in comparison to the climate chamber seemed not to affect the flowering timing nor pollen characteristics. It cannot be ruled out that there are still drivers that have not been accounted for but still influence flowering in the climate chamber (Wolkovich et al. 2012).

The taken twig samples from different harvesting dates were able to flower in the climate chamber/window sill. Flowering in the climate chamber/window sill occurred 5–10 days after harvesting of the twigs. The respective five catkins per twig sample produced a sufficient amount of pollen (>10 mg) for further analysis in the laboratory. These results confirm that the twig methods in climate chambers can be a proxy for outdoor manipulations and can therefore be used for various experimental setups in pollen research (different climatic scenarios for temperature, air humidity, ozone, CO_2) (Primack et al. 2015; Vitasse and Basler 2014).

A recent study by Ettinger et al. (2020) pointed out the dominant influence of winter temperatures on spring phenology and how manipulation experiments can contribute to its further exploration. Branch experiments can simulate variation in chilling length using different harvest dates prior to actual flowering or higher temperatures in the climate chamber/window sill can be used to study the effect of accelerated warming (forcing) (see e.g. Menzel et al. 2020a, b).

Conclusion

The twig method in climate chambers has been already well established for observations on phenology, budburst, and leaf unfolding. The presented study could illustrate that the twig method in climate chambers is also well applicable for investigations on flowering and pollen characteristics. In situ comparisons on outdoor shrubs/trees revealed no significant difference between male catkins from cut and uncut twigs in their time of flowering and pollen characteristics. The addition of different fertilizers during the irrigation of the cut twigs did not show a significant effect on the vitality of the inflorescences nor the pollen characteristics. Twigs from hazel, birch, and alder were cut in different intervals before natural flowering and then kept under controlled conditions in the climate chamber/window sill for flowering. It could be seen that accelerating warming influences flowering and the pollen characteristics catkin length, pollen emission per catkin, and protein content. The twig samples from all cutting dates were able to produce an appropriate amount of pollen for the subsequent laboratory analysis. This shows that the twig method in climate chambers can be used as a proxy for outdoor manipulations in pollen research.

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Author contribution Conceived and designed the study: H.S.J., F.Z., A.M.; carried out the experiment: H.S.J.; analyzed the data: H.S.J.; writing—original draft preparation: H.S.J.; writing—review and editing: H.S.J., F.Z., A.M

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Declarations

Conflict of interest The authors declare no competing interests.

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