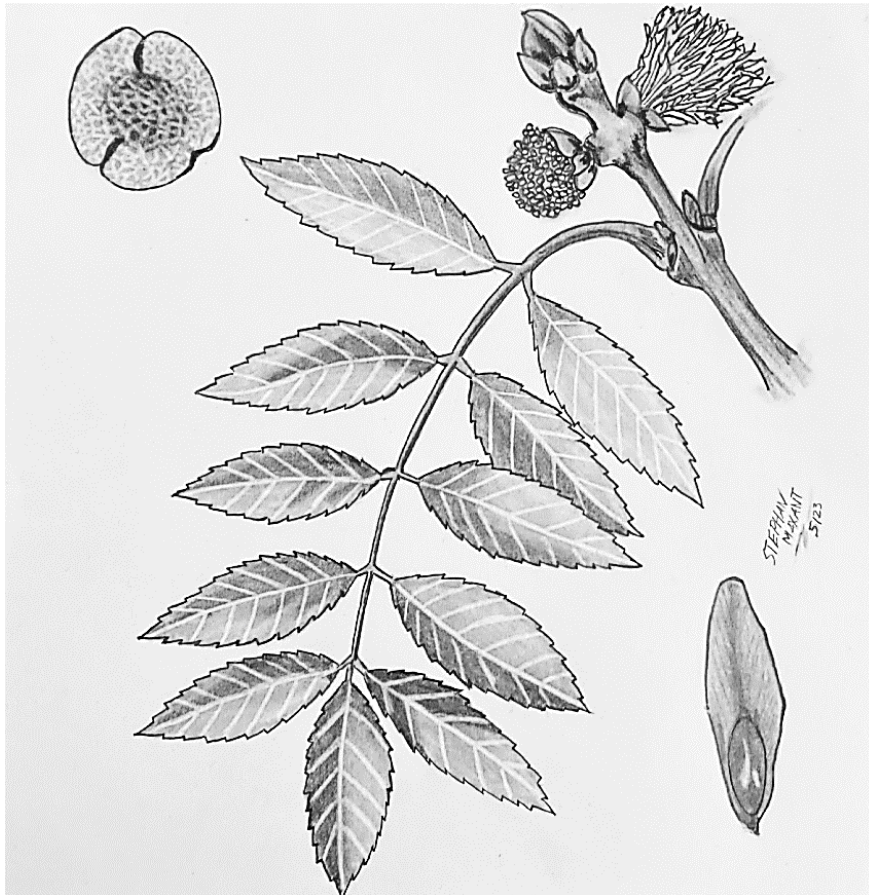


*Influence of the ash dieback on (effective) pollen transport
and reproductive ecology of *Fraxinus excelsior* L.*



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Abstract

The existence of the common ash (*Fraxinus excelsior* L.) is acutely threatened by ash dieback, caused by the fungus *Hymenoscyphus fraxineus*. The disease may lead to reduced pollen flow due to crown damage and tree mortality, which affects the reproductive ecology of *Fraxinus excelsior* L. However, sexual reproduction in forest trees plays an essential role not only in species conservation and adaptive potential, but also in natural selection and the promotion of genetic diversity. The impending risk of reduced gene flow is therefore of serious concern and threatens the conservation of this valuable tree species. Therefore, it is critical to advance research on the effects of ash dieback on (effective) pollen transport and reproductive ecology of *Fraxinus excelsior* L. These results can be used to support forest management, such as maintaining ash genetic diversity and promoting the development of natural resistance. Therefore, this study will conduct extensive research in two seed orchards and one floodplain forest in Southern Germany from 2018 to 2021. The main objective of this dissertation was to improve our understanding of gene flow patterns by investigating (effective) pollen dispersal, pollen production and pollen and seed quality of ash trees in relation to their health status.

The main research questions were:

- (1) How does ash (effective) pollen transport vary with stand density, ash abundance and meteorology?
- (2) Is there a link between mating success and damage to the paternal tree?
- (3) Does the health of the ash trees affect pollen and seed quality, as well as pollen quantity?

To answer these questions, different methods were used in the study areas, which differed in topography and degree of stocking. Therefore, not all parameters were examined at every site and in every year, depending on the issue concerned. The health status of the ash trees was assessed every year in July using a six-level vitality score. In addition, phenological observations were made annually in spring according to the BBCH code to determine different phenological stages such as beginning of flowering, full flowering, flower wilting and end of flowering.

Aerobiological pollen measurements were carried out in the two orchards in 2019 and 2020 using self-constructed gravimetric pollen traps of the Durham type. One of the traps was placed in the center of each orchard and up to five additional traps were placed at a maximum distance of 500 m from the orchard. Five vaseline-coated slides were attached to each trap (one in each cardinal direction and one horizontally) to measure pollen transport (N=4) and weekly cumulative pollen position (N=1). To study the influence of meteorological parameters, a climate station was placed in each orchard. In addition, other anemometers or data loggers for air temperature and relative humidity were installed.

For paternity analyses, cambium samples were collected for genotyping from branches and stumps of 251 ash trees in a seed orchard and floodplain forest. In addition, seeds were collected from 12 parent trees per site with different health status. Genetic analyses were performed to determine paternal trees using nuclear microsatellites. Paternities were assigned using the likelihood model implemented in Cervus 3.0.7 software.

Pollen viability of ash trees with different health status were studied at all three sites. Pollen production in the two seed orchards and seed quality in one orchard and the floodplain forest. For this purpose, inflorescences of 105 ash trees (pollen production), pollen of 125 ash trees (pollen viability) and seeds of 53 ash trees (seed quality) were collected. Pollen production was estimated from flower

to tree level and pollen viability was determined using the TTC (2,3,5-triphenyltetrazolium chloride) test. In addition, pollen storage experiments were carried out to investigate the influence of meteorological factors on pollen viability. Pollen were stored for various periods at different temperatures and humidity conditions and their viability was determined. Seeds were analyzed for quantity and quality (solid, hollow grains, insect damage and germination) and seed quality was determined by both stratification and the TTC test.

The results on aerobiological pollen transport showed that pollen transport is related to meteorology, phenological development and the topography of the study site. The Schorndorf orchard, located on a slope, showed higher pollen levels at the downslope traps, likely due to the transport of pollen by cold air flow. The prevailing wind direction often corresponded with the compass direction in which the highest pollen deposition was measured. When analyzing the pollen data for individual traps, the highest pollen levels were measured outside the orchards in 2019, a year with low flower development. In contrast, most pollen were collected inside the orchard in the pollen-rich year 2020. This suggests that trees outside the orchard can potentially contribute more to pollination in a poor pollen year. In Emmendingen, minor difference in pollen quantity was found between traps at 1.5 m and 5 m height, but there were temporal differences indicating vertical variation in pollen availability. Ash pollen were found to be transported over a distance of more than 400 m, with the amount of pollen decreasing with increasing distance. About 50 % less pollen were measured at a distance of 200 m, but even at a distance of 500 m more than 10 % pollen were still detected.

The studies on effective pollen transport showed similar results: the average distance between father and mother tree was 76 m in the orchard and 166 m in the floodplain forest, with pollination success decreasing considerably with increasing distance. Despite the dense tree cover, the longest distances (> 550 m) were recorded in the floodplain forest. Based on this and the fact that 66.5 % cross-pollen input was recorded in the seed orchard, it can be assumed that pollination does not only involve local sources. However, self-pollination generally did not have a substantial effect on ash reproduction. In terms of health status, both healthy and slightly damaged parent trees showed similar mating success. However, in severely damaged trees almost no flowering could be documented. In general, it was demonstrated that both healthy and slightly diseased fathers sired more offspring than severely diseased ash trees.

The effect of ash dieback on pollen production showed no significant difference between healthy and diseased ash trees, although 53 % of severely diseased male ash trees did not produce flowers. In terms of pollen viability, there was a tendency for diseased ash trees to have less viable pollen than healthy ash trees. In addition, the viability experiments revealed a strong influence of prevailing temperatures on pollen viability, which decreased more rapidly under warmer conditions. There was also no statistical difference in seed quality between ash trees of different health status. However, non-viable seeds were often heavily infested with insects.

In summary, the studies show that a variety of factors influence the reproductive ecology and (effective) pollen transport of ash trees. However, the fact that severely affected ash trees are linked to a reduced flower production and lower pollen quality suggests that as health deteriorates, reproductive capacity also declines. It is therefore likely that severely diseased ash trees have a limited ability to pass on their genes to the next generation. Paternity analyses also suggest that high vigor has a positive effect on reproductive success and that pollen transport can occur over long distances. As susceptibility to ash dieback can be passed on from parents to offspring, these reactions to ash dieback may make ash populations less susceptible in the future.

Zusammenfassung

Die Gemeine Esche (*Fraxinus excelsior* L.) ist durch das Eschentriebsterben, ausgelöst durch den Pilz *Hymenoscyphus fraxineus*, akut in ihrer Existenz bedroht. Die Krankheit kann durch Kronenschäden und der Mortalität von Bäumen zu einem verringerten Pollenfluss führen, was sich auf die Reproduktionsökologie von *Fraxinus excelsior* L. auswirkt. Die geschlechtliche Fortpflanzung von Waldbäumen spielt jedoch nicht nur bei der Arterhaltung und dem Anpassungspotenzial eine essentielle Rolle, sondern auch für die natürliche Selektion und die Förderung der genetischen Vielfalt. Die mögliche Gefahr eines verminderten Genflusses ist daher sehr besorgniserregend und bedroht den Erhalt dieser wertvollen Baumart. Daher ist es von entscheidender Bedeutung, die Forschung zu den Auswirkungen des Eschentriebsterbens auf den (effektiven) Pollentransport und die Fortpflanzungsökologie von *Fraxinus excelsior* L. voranzutreiben. Diese Ergebnisse können zur Unterstützung der Waldbewirtschaftung genutzt werden, bspw. um die genetische Vielfalt der Esche zu erhalten und die Entwicklung natürlicher Resistenzen zu fördern. Daher wurden im Rahmen dieser Studie von 2018 bis 2021 umfangreiche Untersuchungen in zwei Samenplantagen und einem Auenwald in Süddeutschland durchgeführt. Dabei war das Hauptziel dieser Dissertation, unser Verständnis von Genflussmustern zu verbessern, indem die (effektive) Pollenausbreitung, die Pollenproduktion sowie die Pollen- und Samenqualität von Eschen in Abhängigkeit von ihrem Gesundheitszustand untersucht wurde.

Die wichtigsten Forschungsfragen lauteten:

- (1) Wie variiert der (effektive) Pollentransport der Esche in Abhängigkeit von der Bestandsdichte, dem Eschenvorkommen und der Meteorologie?
- (2) Gibt es einen Zusammenhang zwischen dem Paarungserfolg und der Schädigung des Vaterbaums?
- (3) Beeinflusst der Gesundheitszustand der Eschen die Pollen- und Samenqualität sowie die Pollenmenge?

Zur Beantwortung dieser Fragen wurden verschiedene Methoden in den Untersuchungsgebieten angewandt, die sich in Topographie und Bestockungsgrad unterscheiden. Je nach Fragestellung wurden daher nicht alle Parameter an jedem Standort und in jedem Jahr untersucht. Die Beurteilung des Gesundheitszustandes der untersuchten Eschen erfolgte jährlich im Juli anhand eines sechsstufigen Vitalitätsschlüssels. Zudem wurden jährlich im Frühjahr phänologische Beobachtungen nach dem BBCH-Code durchgeführt, um verschiedene phänologische Stadien wie Blühbeginn, Vollblüte und Blütenwelke zu bestimmen.

Die aerobiologischen Pollenmessungen wurden in den Jahren 2019 und 2020 mit selbstgebauten gravimetrischen Pollenfallen vom Typ Durham an den beiden Plantagen durchgeführt. Jeweils eine der Fallen wurde im Zentrum der Plantage platziert und bis zu fünf weitere Fallen wurden in einer Entfernung von maximal 500 m von der Plantage aufgestellt. An jeder Falle wurden fünf mit Vaseline beschichtete Objektträger angebracht (einer in jeder Himmelsrichtung und einer horizontal), um wöchentlicher den Pollentransport (N=4) und die kumulative Pollenposition (N=1) zu messen. Um den Einfluss der meteorologischen Parameter zu untersuchen, wurde eine Klimastation in jeder Plantage aufgestellt. Zusätzlich wurden weitere Anemometer bzw. Datenlogger für Lufttemperatur und relative Luftfeuchtigkeit installiert.

Für die Vaterschaftsanalysen wurden von 251 Eschen Kambiumproben von Zweigen und Baumstümpfen zur Genotypisierung in einer der Samenplantagen und im Auwald entnommen. Zusätzlich wurden Samen von 12 Elternbäumen pro Standort mit unterschiedlichem Gesundheitszustand gesammelt. Zur Bestimmung der Vaterbäume wurden genetische Analysen unter Verwendung von

Kernmikrosatelliten durchgeführt. Die Vaterschaften wurden anhand des in der Software Cervus 3.0.7 implementierten Wahrscheinlichkeitsmodells zugewiesen.

Die Pollenviabilität von Eschen mit unterschiedlichem Gesundheitszustand wurde an allen drei Standorten untersucht. Die Pollenproduktion in den beiden Samenplantagen und die Samenqualität in einer Plantage und im Auwald. Zu diesem Zweck wurden Blütenstände von 105 Eschen (Pollenproduktion), Pollen von 125 Eschen (Pollenviabilität) und Samen von 53 Eschen (Samenqualität) gesammelt. Die Pollenproduktion wurde von der Blüten- bis zur Baumebene geschätzt und die Pollenviabilität mittels des TTC-Test (2,3,5-Triphenyltetrazoliumchlorid) bestimmt. Zusätzlich wurden Experimente zur Pollenlagerung durchgeführt, um den Einfluss meteorologischer Faktoren auf die Lebensfähigkeit der Pollen zu untersuchen. Dabei wurde der Pollen über verschiedene Zeiträume bei unterschiedlichen Temperatur- und Feuchtebedingungen gelagert und ihre Lebensfähigkeit bestimmt. Die Samen wurden hinsichtlich Quantität und Qualität (Voll-, Hohlkörner, Insektenschäden, Keimfähigkeit) analysiert, dabei erfolgte die Bestimmung der Samenqualität sowohl durch Stratifikation als auch mit dem TTC-Test.

Die Ergebnisse zum aerobiologischen Pollentransport zeigten, dass der Transport von Pollen mit der Meteorologie, der phänologischen Entwicklung und der Topographie des Untersuchungsgebietes zusammenhängt. Die Plantage in Schorndorf, die an einem Hang liegt, wies an den hangabwärts gelegenen Fallen höhere Pollenwerte auf, was wahrscheinlich auf den Transport von Pollen durch Kaltluftabflüsse zurückzuführen ist. Die vorherrschende Windrichtung entsprach häufig der Himmelsrichtung, in der die höchste Pollenablagerung gemessen wurde. Bei der Analyse der Pollendaten für einzelne Fallen wurde festgestellt, dass im Jahr 2019, einem Jahr mit geringer Blütenentwicklung, die höchsten Pollenmengen außerhalb der Plantagen gemessen wurden. Im pollenreichen Jahr 2020 wurde die größte Pollenmenge hingegen innerhalb der Plantage gefangen. Dies deutet darauf hin, dass in einem pollenarmen Jahr Bäume außerhalb der Plantage möglicherweise mehr zur Bestäubung beitragen können. In Emmendingen wurden nur geringe Unterschiede in der Pollenmenge zwischen Fallen in 1,5 m Höhe und 5 m Höhe festgestellt, jedoch gab es zeitliche Unterschiede, die auf vertikale Schwankungen in der Pollenverfügbarkeit hindeuteten. Es wurde festgestellt, dass Eschenpollen über eine Entfernung von mehr als 400 m transportiert werden können, wobei die Pollenmenge mit zunehmender Entfernung abnimmt. Bereits bei einer Entfernung von 200 m wurden etwa 50 % weniger Pollen gemessen, jedoch wurden selbst bei einem Abstand von 500 m noch mehr als 10 % der Pollen erfasst.

Ähnliche Ergebnisse zeigten auch die Untersuchungen zum effektiven Pollentransport: So lag die durchschnittliche Entfernung zwischen Vater- und Mutterbaum innerhalb der Plantage bei 76 m und im Auwald bei 166 m, wobei der Bestäubungserfolg mit zunehmender Entfernung deutlich abnahm. Die größten Distanzen (> 550 m) konnten trotz der dichten Baumdeckung im Auwald detektiert werden. Aufgrund dessen und der Tatsache, dass in der Samenplantage 66,5 % Fremdpolleneintrag zu verzeichnen war, kann davon ausgegangen werden, dass die Bestäubung nicht nur durch lokale Quellen erfolgt. Die Selbstbestäubung hatte jedoch im Allgemeinen keinen wesentlichen Einfluss auf die Reproduktion der Esche. Hinsichtlich des Gesundheitszustandes zeigten sowohl gesunde als auch schwach geschädigte Vaterbäume einen ähnlichen Paarungserfolg. Bei stark geschädigten Bäumen konnte jedoch nahezu keine Blütenbildung dokumentiert werden. Generell konnte nachgewiesen werden, dass sowohl gesunde als auch leicht kranke Väter mehr Nachkommen zeugten als stark erkrankte Eschen.

Die Auswirkungen des Eschentriebsterbens auf die Pollenproduktion zeigte keinen signifikanten Unterschied zwischen gesunden und kranken Eschen, obwohl 53 % der stark erkrankten männlichen Eschen keine Blüten produzierten. Hinsichtlich der Pollenviabilität wurde festgestellt, dass kranke Eschen tendenziell weniger lebensfähige Pollen aufweisen als gesunde Eschen. Zudem konnte bei

den Viabilitätsversuchen ein starker Einfluss der Temperatur auf die Lebensfähigkeit der Pollen festgestellt werden, die unter wärmeren Bedingungen schneller abnahm. Bei der Samenqualität gab es keine statistischen Unterschiede zwischen Eschen mit unterschiedlichem Gesundheitsstatus. Allerdings waren die nicht lebensfähigen Samen oft stark von Insekten befallen.

Zusammenfassend zeigen die Untersuchungen somit, dass eine Vielzahl von Faktoren die Reproduktionsökologie und den (effektiven) Pollentransport der Esche beeinflussen. Vor allem die Tatsache, dass stark befallene Eschen eine geringere Blütenbildung und Pollenqualität aufweisen, deuten jedoch darauf hin, dass mit fortschreitender Beeinträchtigung der Gesundheit auch die Reproduktionsfähigkeit abnimmt. Es ist daher wahrscheinlich, dass stark erkrankte Eschen nur begrenzt in der Lage sind, ihre Gene an die nächste Generation weiterzugeben. Auch die Vaterschaftsanalysen legten dies nahe und zeigten, dass sich eine hohe Vitalität positiv auf den Fortpflanzungserfolg auswirkt und dass der Pollentransport auch über weitere Distanzen stattfinden kann. Da die Anfälligkeit für das Eschensterben von den Eltern an die Nachkommen weitergegeben werden kann, können diese Reaktionen auf das Eschensterben dazu führen, dass Eschenpopulationen in Zukunft weniger anfällig sind.

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1 Introduction

Forests not only play an important role in the global carbon cycle by acting as carbon sinks, they also provide other important ecosystem services, for example they yield highly valued resources like fuels, timber and food, support biodiversity, provide habitats and facilitate the production of sustainable products (Isabel et al. 2020; Balla et al. 2021). However, biotic disturbances, such as the introduction of forest pathogens and pests due to globalization (e.g., fungi, oomycetes, bacteria, viruses, nematodes, and parasitic plants), as well as direct and indirect effects of climate change, have led to intense tree mortality and degradation of forest quality in recent decades (Santini et al. 2013; Goberville et al. 2016; Kautz et al. 2017; Balla et al. 2021; Gašparović et al. 2023). This poses an increasing challenge to the forest industry in terms of ensuring site-appropriate, environmentally meaningful, and economically efficient forest management (Dichtl and Stöger 2020). A major challenge to the adoption of forest management techniques is the lack of knowledge on (locally) changing environmental conditions and the adaptive capacity of forest tree species. However, this knowledge is extremely relevant in view of the long generation times in forested landscapes of more than 100 years (Kölling 2007; Pautasso et al. 2010; McKinney et al. 2014; Isabel et al. 2020). Moreover, the consequences for trophic interactions, biogeochemical cycles, and ecosystem functions are difficult to estimate (Hultberga et al. 2020). To maintain the genetic flexibility and fitness of forest ecosystems, close cooperation between research and forestry operations is required. Thus, early detection and research of abiotic and biotic risks as well as the development of appropriate management strategies could mitigate potential disturbances and protect forest health in the future.

More than 50 % of recent plant diseases are associated with biological invasions (Santini et al. 2013), and rank among the top five drivers of direct global biodiversity loss (Maxwell et al. 2016). Especially the number of plant-damaging fungi in forest trees has multiplied and has already led to severe epidemics (Fisher et al. 2012; Santini et al. 2013; Balla et al. 2021). For example, Dutch elm disease and chestnut blight have nearly wiped out their host species (Santini et al. 2013; Hultberga et al. 2020), while *Phytophthora* root neck rot currently threatens alder (Dichtl and Stöger 2020; Lorenc and Samek 2021). Generally and as a result of repeated tree losses and a decline in progeny, many forest tree populations are assumed to be at risk of serious genetic impoverishment (Santini et al. 2013), and at least 10 % of forest trees worldwide are considered to be at risk of extinction (Goberville et al. 2016).

A similar fate threatens the common ash (*Fraxinus excelsior* L.; family: Oleaceae), whose existence is acutely endangered by ash dieback (Metzler et al. 2012; McKinney et al. 2014; Enderle 2019), a disease caused by the fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya (Baral et al. 2014) (syn. *Hymenoscyphus pseudoalbidus* (Queloz et al. 2011) and its asexual stage *Chalara fraxinea* (Kowalski 2006), native to East Asia. Simulations indicated that the invasive fungus will limit the growth of the common ash (hereinafter called “ash”) to such an extent that within the next 30 years only 25-50 % of the current trees will be found in natural forests in Europe (Coker et al. 2019; George et al. 2022). Seed orchards, which represent selective breeding populations (Fussi et al. 2014) established prior to the onset of ash dieback, are assumed to have a maximum survival rate of only 15 %, as the high number of ash trees in a small area is associated with an even higher infection pressure (Coker et al. 2019). However, the loss of ash is particularly severe due to its valuable wood properties, unique ecophysiology, and climate and drought tolerance (Marigo et al. 2000; Kölling 2007; Pautasso et al. 2013; Enderle et al. 2017; Gašparović et al. 2023).

The tree species, which has been native to Europe for centuries, was planted frequently in the 1990s as a climate-smart tree in the context of forest conversion, and was actively promoted by the forestry sector to economically and ecologically improve mixed forest ecosystems (Enderle et al. 2017; Müller-Kroehling and Schmidt 2019; Bartsch et al. 2020). Its distribution range (Figure 1) includes most of Europe, with the exception of Portugal, Iceland, and Malta, as well as the northernmost (northern

limit: 64° N) and southernmost (southern limit: 37° N in Iran) parts of the continent (Pliūra and Heuertz 2003; Dobrowolska et al. 2011; Douglas et al. 2013; Enderle 2019). In terms of vertical distribution, the limit is 1630 m a.s.l. in the Alps, approx. 1800 m a.s.l. in the Pyrenees, and up to 2200 m a.s.l. in Iran (Marigo et al. 2000; Douglas et al. 2013). The northern limit is assumed to be determined by the amount of available energy required for the annual life cycle (Dobrowolska et al. 2011). The eastern limit is determined by minimum temperatures, while lack of moisture affects the southern and southeastern limits (Dobrowolska et al. 2011). In total, ash only covers about 1-2 % of the forest area in Europe (Skovsgaard et al. 2017; Enderle 2019) and about 2.4 % of the forest area in Germany (Enderle et al. 2017). Despite the small percentage, ash can occur locally in high densities and contributes decisively to the economic performance of stands (Skovsgaard et al. 2017). Ash is particularly important in Southern Germany, where its share of the total mixed forest area in Germany is about 48 % (BWI 2012), and in the United Kingdom and Ireland, where ash is nearly ubiquitous except in the northernmost parts (Enderle 2019).

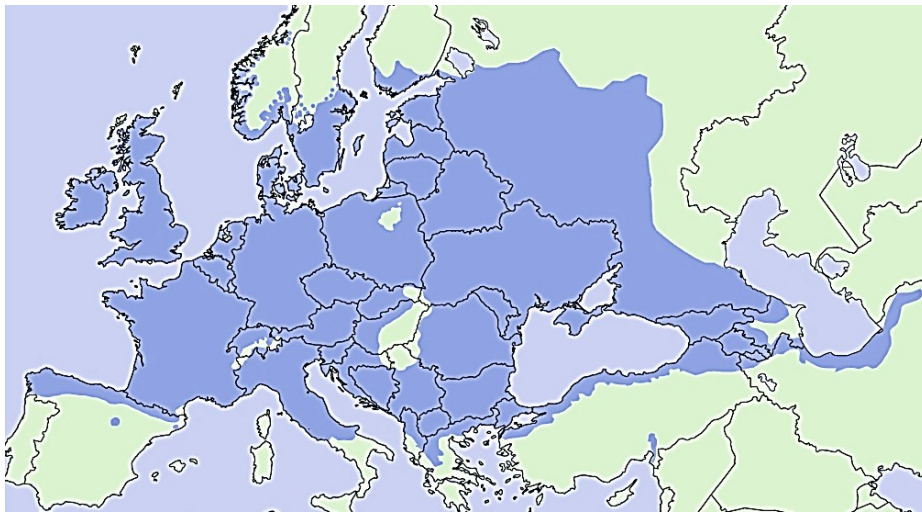


Figure 1: Distribution range of common ash (*Fraxinus excelsior* L.) in Europe (Pliūra and Heuertz 2003).

The ash is capable of thriving on calcareous, loamy-clayey and nutrient-rich soils and can occur in summer-warm and comparatively dry climatic regions but is also known for its high flood tolerance (Wardle 1961; Metzler et al. 2012; Enderle et al. 2015). Therefore, it is one of the dominant species in floodplain (Dufour and Piégay 2008; Stammel et al. 2012; Mitchell et al. 2014a) and canyon forests (Weber-Blaschke et al. 2008; Jedicke 2015). Furthermore, due to their vast and dense root systems, ash trees are frequently planted to stabilize slopes and embankments, minimizing soil erosion (Rigling et al. 2016). Another advantage of ash trees is that their leaves and roots are readily decomposable, and they absorb minimal nutrients from their leaves before shedding them (Mitchell et al. 2014b). This increases the pH of the soil, which reduces acidification and promotes soil life (Jacob et al. 2009; Langenbruch et al. 2012). In addition, its nutrient-rich bark provides a microhabitat for numerous mosses, lichens, fungi and insects, some of which are already on the Red List (Mitchell et al. 2014b; Hultberga et al. 2020). In total, approximately 1,000 species are estimated to be associated with ash or ash woodlands (Mitchell et al. 2014a; Mitchell et al. 2014b), with for example 44 species obligately associated with ash in the UK (Mitchell et al. 2014a) and 52 species in Sweden (Hultberga et al. 2020). Furthermore, Hultberga et al. (2020) found that many of the species associated with ash are also associated with elm (*Ulmus glabra*), which is threatened by elm mortality. Upon their calculation up to nine additional tree species (*Acer platanoides* L., *Betula* spp., *Corylus avellana* L., *Fagus sylvatica* L., *Picea abies* (L.) H. Karst, *Pinus sylvestris* L., *Populus tremula* L., *Sorbus aucuparia* L., and *Quercus* spp.) would be needed on a site to maintain all of the non-obligate species from

ash and elm. Thus, ash plays a key role in deciduous forest ecosystems and has a substantial impact on the habitat through interactions and nutrient cycling. Moreover, the ash tree has had a high cultural and economic value since ancient times (Pratt 2017; Rutjes 2017). Thus, as a symbol of eternal life, the ash is often planted in front of churches in Scandinavia (FNR 2022) and is often used for furniture, veneer, tools or sports equipment due to its rapid vertical growth and the high stability and elasticity of the wood (Dobrowolska et al. 2011; Pautasso et al. 2013).

However, the ongoing fragmentation of ash trees (McKinney et al. 2014; Semizer-Cuming et al. 2017) as well as the associated risk of reduced gene flow (Sork and Smouse 2006; Frankham et al. 2012) are serious concerns for the conservation of this valuable tree species. To date, studies on ash dieback have focused primarily on the areas of resistance and mortality rates of ash trees, as well as on the pathogen itself (see Chapter 2.2). In order to preserve the genetic diversity of ash, research on the influence of ash dieback on (effective) pollen transport and reproductive ecology is also urgently needed. These results can be used to support forest management, to promote the development of natural resistance, as only targeted measures can ensure that ash trees will still be part of our forests and landscapes in the future and will be able to fulfill their valuable ecological and economic functions. The present thesis aims to contribute to this effort and to shed more light on the effects of ash dieback on reproductive ecology and pollination success.

2 Current state of research

2.1 Reproduction ecology and influencing factors

2.1.1 Reproduction ecology of forest trees

The reproduction of forest trees not only contributes to the conservation of species, but also serves to maintain the genetic diversity of future generations and the distribution of species (Smith 1981; Jump et al. 2009; Isabel et al. 2020; Wang et al. 2022). It also promotes the adaptive potential of species and is a central factor in natural selection (Smith 1981; Jump et al. 2009; Wang et al. 2022). Thus, in a healthy ecosystem, the individuals that reproduce as part of the evolutionary adaptation process are those that have the best adapted traits under the given environmental conditions and requirements, and pass these traits on to the next generation (Fussi et al. 2014). The two most important mechanisms for gene flow within and between populations are pollen and seed dispersal (Goto et al. 2006; Fussi et al. 2014; Breygina et al. 2021):

In general, sexual reproduction in forest tree species occurs by anemophilic pollination of female flowers with male pollen (LaDeau and Clark 2006; Fussi et al. 2014; Larue et al. 2021). To increase the likelihood that a male pollen grain will encounter a female flower and to ensure successful fertilization, species that rely on wind pollination produce a much larger quantity of flowers and pollen than insect-pollinated species (Holsinger and Steinbachs 1997; Dellinger 2020; Timerman and Barrett 2020). For this purpose, buds or catkins are formed in the previous year before the tree enters winter dormancy (Rohde and Bhalerao 2007). In spring, the flowering and pollen dispersal occurs when day length increases and a critical species-specific temperature sum is reached (Tooke and Battey 2010). Thereby, the pollen vectors act stochastically in most cases (Bochenek and Eriksen 2011). This means that the order in which pollen are released and reach the female flower has an influence on the pollination success (Burkhardt et al. 2009; Bochenek and Eriksen 2011). If a pollen grain reaches a female flower, it germinates and forms a pollen tube that grows into an ovule (Breygina et al. 2021).

However, if more pollen grains reach the female flowers at the same time than ovules are available, competition between male gametophytes may occur. The speed of germination of pollen grains and

sperm transport is influenced by the fitness of the pollen, which might result in delayed fertilization (Bochenek and Eriksen 2011). Thus, it is hypothesized that increased competition among pollen grains may lead to improved offspring quality (Marshall et al. 2007; Lankinen et al. 2009). Another important feature of successful pollination is the synchrony of flowering times. Only if the flowering times of the male and female flowers overlap and there is enough viable pollen available, the male pollen can fertilize the female flower (Dowding 1987; Mondal et al. 2019). The natural viability of pollen from forest tree species ranges from a few days to several weeks, depending on the species and location (Dietze 1973). However, several studies have shown that the quantity and quality of pollen produced can be influenced by both genetic (Rousi et al. 2019; Ranpal et al. 2022) and external environmental factors, such as air pollutants (CO, CO₂, SO₂, NO₂, O₃) (LaDeau and Clark 2006; Rezanejad 2007; Darbah et al. 2008; Duro et al. 2013) or weather-related variables (D'Amato et al. 2007; Zinn et al. 2010; Bonofiglio et al. 2013; Rojo et al. 2015b; Luschkova et al. 2022; Zhang and Steiner 2022). For example, CO, SO₂, NO₂ and O₃ were often associated to negative effects on pollen viability (Duro et al. 2013) or pollen production (Darbah et al. 2008), while CO₂ was often linked to a positive effect (LaDeau and Clark 2006; Darbah et al. 2008).

The fusion of the gametes finally results in the new genotype of the seed, which combines the genetic information from both the mother tree and the father tree (Bochenek and Eriksen 2011; Fussi et al. 2014). Years with above-average flower and pollen production increase the chances of successful pollination, which in turn has a direct impact on seed production and influences mast years and mast failures (Satake and Iwasa 2002; LaDeau and Clark 2006; Mangla and Gupta 2015). Mastings is a phenomenon that is particularly common in wind-pollinated forest trees. It is characterized by a large variation in annual seed production, with synchronization of populations observed over large geographical areas (Fernández-Martínez et al. 2012; Shibata et al. 2020; Koenig 2021). A similar behavior has already been observed for flower and pollen production (Gassner et al. 2019). Thus, pollen and flower production could be a factor explaining why seed production of many forest trees varies greatly from year to year.

2.1.2 Peculiarities of ash

Ash is also a wind-pollinated deciduous tree that can reach a maximum height of 40 m and a trunk diameter of up to 150 cm at the age of 90-120 years (Douglas et al. 2013; Thomas 2016; Westergren et al. 2020). The crown of the tree is usually elongated, irregular, and heavily branched (Douglas et al. 2013). Sexual maturity of the ash occurs at the age of 15-30 years in open stands and at the age of 30-35 years in closed stands (Roloff and Pietzarka 1997; Douglas et al. 2013). Its purple flowers are arranged in small, inconspicuous axillary panicles (inflorescences) and appear before the pinnate leaves (Thomas 2016; Westergren et al. 2020). Each inflorescence usually produces over 100 flowers (Thomas 2016). However, ash trees are also subject to a natural cycle of pollen-poor and pollen-rich years (Kelly 1994; Gassner et al. 2019). In Switzerland for example, pollen-rich years were observed in 2003, 2005, 2007, 2009, 2013, 2015, and 2018 (Gassner et al. 2019). In temperate climates, ash flowering usually begins in mid–end of March and ends in the beginning to the middle of May (Albert et al. 2013, 2013; Douglas et al. 2013; Westergren et al. 2020), with male flower buds opening a few days earlier (Albert et al. 2013). In addition, the ash tree is very sensitive to spring frosts, which can lead to freezing of flowers, leaves and buds (Kerr and Boswell 2001; Thomas 2016).

It is noteworthy that the ash is polygamous and therefore trioecious (Figure 2). This means that the inflorescences can be female, male or hermaphrodite (Roloff and Pietzarka 1997; Albert et al. 2013; Douglas et al. 2013; Saumitou-Laprade et al. 2018). In this case, the male ash trees produce pollen, the female trees seeds and the hermaphrodite trees seeds and pollen (Albert et al. 2013). In addition,

gender expressions may vary from year to year (Douglas et al. 2013; Westergren et al. 2020). However, Albert et al. (2013) assume that this expression is genetically determined and stabilizes as the tree ages. In general, this complex reproductive system is found in only 3.6 % of all flowering plants and is therefore very rare (Albert et al. 2013). Due to hermaphroditism, ash is generally self-pollinated, but Saumitou-Laprade et al. (2018) suggested that self-pollinated seeds are not viable for development due to inbreeding depression, and thus the species is actually subdioecious (functionally dioecious).

If the female inflorescences have been successfully pollinated, the seeds will develop as a product of sexual reproduction. The mature wingnuts are in turn carried by the wind and rest for two to six winters before germinating (Douglas et al. 2013; Westergren et al. 2020). Despite a low germination rate of about 58-65 % (Roloff and Pietzarka 1997; Schirmer 2002), ash trees can achieve regeneration rates of up to 150,000 individuals per hectare under good site conditions due to their very high seed production (Tabari and Lust 1999; Dobrowolska et al. 2011). The seedlings prefer low light and are very tolerant of shade, whereas mature ash trees have a much higher light demand, which promotes height growth (Roloff and Pietzarka 1997; Schütz 2004; Petritan et al. 2007).

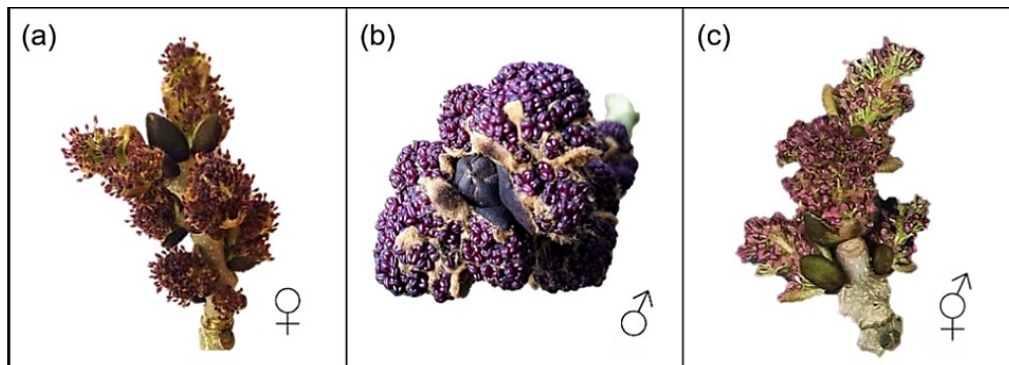


Figure 2: Inflorescences of *Fraxinus excelsior* L.: (a) female, (b) male, and (c) hermaphrodite. Leaves develop from the terminal bud. Source: (a) and (b) own image; (c) excerpt from <http://www.aurbaniak.de/Bilder/Fraxinus-excelsior.jpg> (17.05.2023).

2.2 Ash dieback

Ash dieback, the greatest threat to ash to date (Skovsgaard et al. 2017; Gašparović et al. 2023), is an important factor that could negatively affect the reproductive processes and adaptive potential of the species. Thus, Kozłowski already noted in 1971 that the vigor and vitality of forest trees correlated with their ability to produce flowers and fruit.

2.2.1 Distribution and pathogenesis

Meanwhile, the invasive fungus *H. fraxineus*, which causes ash dieback, is in almost all natural ash ranges present and has led to severe population declines (Enderle 2019; Hultberga et al. 2020; Gašparović et al. 2023). Figure 3 shows the spread of ash dieback since the first symptoms were observed (Gašparović et al. 2023). In general, the proportion of ash trees that are less susceptible to ash dieback is thought to be very small, varying from 10-20 % (Westergren et al. 2020) to 1-5 % (McKinney et al. 2014; Rigling et al. 2016; Enderle 2019).

The pathogen originates from Far East Asia, where the fungus lives in symbiosis with Manchurian ash (*F. mandshurica*) without causing infection symptoms in this species, and decomposes its litter (Drenkhan et al. 2014; Inoue et al. 2019). It is likely that a few individuals of the fungus were introduced to Europe via imported Asian ash nursery stock in the Baltic countries at the end of the 20th century (Drenkhan et al. 2014; Gross et al. 2014; Hultberga et al. 2020). The fungi then spread rapidly in space, both by importation and by wind dispersal of the ascospores in the air, at 30-70 km per year (Børja et al. 2017; Enderle 2019; George et al. 2022). The first signs of the disease were observed in Poland as early as 1992, but it was not possible to identify the causative agent at that time (Przybyl 2002; Kowalski 2006; Timmermann et al. 2011). In the early 2000s, the first symptoms of ash dieback were detected in Germany and Sweden (Barklund 2005; Hultberga et al. 2020). However, the pathogen itself was not identified as a new anamorphic form of the fungus until 2006 (Kowalski 2006), as the morphologically almost identical but genetically different fungus *Hymenoscyphus albidus* has been previously known as a harmless saprotroph in Europe (McKinney et al. 2014).

The fungus *H. fraxineus* is present for most of the growing season and produces 1 to 3 mm fruiting bodies on the previous year's leaf rachis on the soil (Timmermann et al. 2011; Lenz and Mayer 2016). It releases spores between late spring and autumn, with particularly high spore numbers in the summer months. (Timmermann et al. 2011; Lenz and Mayer 2016). The ascospores of the fungus, which are dispersed by the wind, first attack the leaf rachis and leaves of the trees and then gradually spread to the shoots and wood of the ash trees. As a result, the shoots and, as the disease progresses, the entire tree dies in most cases due to the disruption of nutrient and water transport (Kowalski and Holdenrieder 2009; Timmermann et al. 2011; Pautasso et al. 2013; Cleary et al. 2016; Harper et al. 2016). Symptoms include substantial loss of leaves and dead shoot tips, wilting, bark necrosis on stems and discolouration of the wood (Lenz et al. 2012a). From October onwards, the infected leaves fall to the ground, where the fungus survives on the new fallen leaf rachis during the winter and produces fruiting bodies again in the spring (Rigling et al. 2016; Gašparović et al. 2023). In contrast, the role of *Chalara fraxinea* (asexual stage of *H. fraxineus*) remains controversial (Gross et al. 2014). Thus, it is still unclear whether its function is limited to reproduction due to the conidia, which are often regarded as spermatozoa (Gross et al. 2014), or whether it also contributes to the increase in inoculation pressure due to its ability to germinate (Fones et al. 2016).

Ash dieback can cause severe damage to trees of any age, but young trees and ash trees in moist locations are particularly susceptible (Marçais et al. 2017; Skovsgaard et al. 2017). This is probably because the fungus thrives in temperatures around 20 °C and high humidity (Grosdidier et al. 2018). In addition, forestry has often responded to ash dieback in old-growth forests by removing ash trees to prevent timber degradation (Pliūra et al. 2017; Enderle 2019), which has led to an almost six-fold increase in felling in Germany, Austria and Denmark since 2008 (McKinney et al. 2014; Heinze et al. 2017; ForstBW 2018). As the disease is particularly lethal in young plants, it is likely that the proportion of ash trees will continue to decline in future generations (Timmermann et al. 2011). In Lithuania, the population of mature ash trees has already declined to such an extent that the conservation of the species and genetic diversity is threatened (Pliūra et al. 2017; Enderle 2019). In Europe, the ash tree is listed as a near threatened (NT) species on the Red List (Rivers et al. 2019); in Sweden it is even considered as vulnerable (Hultberga et al. 2020), in Albania as endangered, in Hungary as serious endangered and in Serbia and Montenegro as rare/endangered (Khela 2013). However, hope is given by the fact that experimental tests showed that the growth of the fungus is limited at temperatures of more than 30 °C, and the fungus was more sensitive to heat in ash tissue than in pure cultures (Hauptman et al. 2013). Thus, the fungus could be pushed back to northern latitudes in the future by the progressive warming associated with climate change, resulting in a partial spatial decoupling of host and pathogen (Goberville et al. 2016).

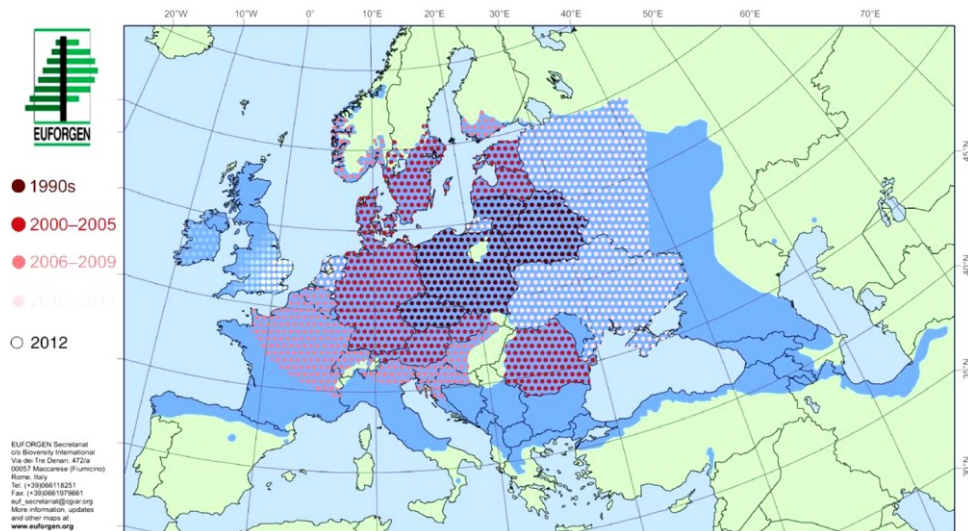


Figure 3: Spread of ash dieback since the first observed symptoms (Gašparović et al. 2023).

2.2.2 Effect of ash dieback on reproduction

Current studies indicate that resistance to ash dieback is not linked to the population or source level, but to the individual level (McKinney et al. 2014; Enderle et al. 2017). Differences in susceptibility between individuals have been observed across Europe (McKinney et al. 2014). These differences have a distinct genetic component that is inherited from parents to offspring (McKinney et al. 2011; Semizer-Cuming et al. 2019). The influence of heritability is estimated to be high, at around 50 % (McKinney et al. 2014; Enderle et al. 2015). Lobo et al. (2015) showed that progeny of low-susceptibility mother clones developed less necrosis compared to progeny of susceptible clones, and crown damage caused by natural infection was much weaker. In addition, the development of resistance may also involve epigenetic effects in individuals infected by the fungus (Sollars and Buggs 2018). In this context, trees with minor or no damage are repeatedly found in the midst of severely damaged or dead trees. These partial resistances are related to very low disease levels in the trees. Individual branches may be affected, but the plant will tolerate the disease and survive. Individuals with less than 30 % leaf loss are considered healthy and suitable for genetic conservation or breeding programs (Lenz et al. 2012a; McKinney et al. 2014). Nevertheless, Fussi et al. (2016) showed that even under infestation pressure, clones of old ash trees that appeared to be resistant can become infected within two years. However, after five years of infestation pressure, the situation has stabilized (5–10 % of grafted plants were asymptomatic or only slightly damaged) and some clones can be considered healthy after eight years of observation (Fussi 2020). Furthermore, Fussi and Konnert (2014) showed that heterozygous individuals appear less susceptible to ash dieback than homozygous individuals (Fussi and Konnert 2014). Therefore, the breeding of tolerant trees could be a great opportunity for the ash tree in the future and could contribute to the conservation of genetic diversity.

To increase the proportion of healthy young ash trees, effective gene flow between resistant or partially resistant ash trees is required (Semizer-Cuming et al. 2017; Fussi 2020). However, McKinney et al. (2014) and Semizer-Cuming et al. (2017) suggested that ongoing fragmentation of ash populations is weakening pollen flow between wind-pollinated trees and consequently reducing gene flow. The consequence would be a restriction of genetic diversity and thus, through reduced natural selection, a negative impact on the adaptive potential of future generations (Sork and Smouse 2006; Fussi and Konnert 2014; McKinney et al. 2014). Furthermore, in their study of genetic connectivity in plant populations, Sork and Smouse (2006) indicated that the ability to withstand fragmentation

depends on the extent of gene flow and the diversity of the pollen pool. The relationship of individuals in populations can be studied by genetic analysis using microsatellites (Wang 2004; Jones and Wang 2010; Karaket and Poompuang 2012). Thus, Heuertz et al. (2003) investigated the effective pollen transport of ash using DNA-based paternity analysis in Romania, Bacles and Ennos (2008) in Scotland, Thomasset et al. (2014) in Ireland, and Semizer-Cuming et al. (2021) in Denmark. In the studies, the distances ranged between 70 and 140 m (Heuertz et al. 2003; Semizer-Cuming et al. 2021) and 2,900 m (Bacles and Ennos 2008). Aerobiological studies, on the other hand, have already provided information on pollen dispersal per se in various anemophilous species. They showed that the dispersal and deposition of pollen is not only influenced by meteorological factors such as temperature, humidity and wind speed, but also by factors such as neighboring vegetation or the size of the tree and crown itself (Puc 2012; Scheifinger et al. 2013; Adams-Groom et al. 2017; Škoparija et al. 2018b).

To date, only Semizer-Cuming et al. (2019) have investigated ash reproductive success with respect to ash dieback. They found a gender-dependent relationship between symptoms and reproductive success in a seed orchard in Denmark. In particular, a significant relationship between fungal infection and mating success was observed in female individuals. In contrast, analysis of male father trees showed no clear relationship between disease status and reproductive success. Interestingly, some male trees were able to reproduce successfully despite high levels of disease (Semizer-Cuming et al. 2019). These observations can be explained partly by gender differences in reproductive investment (Semizer-Cuming et al. 2019) and partly by the effects of stress conditions on reproductive performance (Wada and Takeno 2010). Several studies in dioecious plant species (e.g. *Salix*) have already shown that females devote more resources on reproduction and consequently have higher reproductive costs (Cipollini and Whigham 1994; Antos and Allen 1999; Obeso 2002; Ueno et al. 2007). Therefore, Semizer-Cuming et al. (2019) suggested that crown damage due to ash dieback likely limits the resources available for reproduction in both genders. It is possible that female trees have reduced seed production due to crown damage, as it costs too many resources during the growing season to restore their crown with new shoots and produce reproductive tissue. Male trees, on the other hand, are able to produce pollen and flowers, as this occurs prior sprouting and costs fewer resources (Semizer-Cuming et al. 2019; 2021). In addition, it was found during the forest dieback in the 1980s that stress conditions can trigger an "emergency response" and lead to increased pollen production (Wada and Takeno 2010). Similar effects were also suggested by Gassner et al. (2019), who observed the highest ash pollen emissions one to two years after disease onset in a region.

3 Research gaps and objectives

3.1 Research gaps

Considering the aforementioned state of research, it becomes clear that ash dieback is a major challenge from both an ecological and an economic point of view, which is why research on ash conservation is important and necessary. Despite the proliferation of publications on this topic, few studies have examined the effects of progressive fragmentation of ash populations on gene flow and reproductive success in ash. Ash dieback could affect the reproductive ecology of ash trees, and ash trees could become genetically isolated due to large-scale decline of the species (Sork and Smouse 2006; McKinney et al. 2014; Semizer-Cuming et al. 2017). Thus, fewer potential mates are available for reproduction and the probability decreases that viable pollen will be transported in sufficient quantity and over greater distances from one tree to another by the wind. If populations in natural stands are no longer sufficiently interconnected, this can lead to genetic impoverishment,

negatively affecting the natural resistance of future generations of ash trees (McKinney et al. 2014). In the long term, however, the maintenance of genetic diversity and the adaptability of the species are crucial for the survival of ash in forest stands.

In contrast, spatial limitation of pollen distances is desirable in seed orchards. Pollination with pollen from other ash trees of unknown origin and quality could be detrimental to the production of high quality forest reproductive material. The Forest Reproduction Act (FOVG 2002; BGBl. I p. 1658) provides that seed orchards should be located at a sufficient distance from phenotypically poor stands of the same species or a species that can be crossed with it. As a rule, 400 meters is regarded as sufficient distance, depending on the topography and the stand situation (gGA 2019). Thus, unintended gene flow from other ash populations should be avoided. However, as many seed orchards were planted before the occurrence of ash dieback and selected for growth and stem quality, many of the trees are vulnerable to ash dieback (Enderle et al. 2015). Therefore, as a result of the fragmentation of ash trees, the local pollen will no longer be sufficiently saturated and cross-pollination may occur (Douglas et al. 2013).

As shown, studies of ash pollen dispersal have mostly been indirect, using DNA-based paternity analysis (Heuertz et al. 2003; Bacles and Ennos 2008; Thomasset et al. 2014; Semizer-Cuming et al. 2021). However, there are drawbacks to this method. Due to the widespread distribution of ash trees and the large possible range of pollen transport, it is almost impossible to sample all relevant trees that represent a potential pollen source for reproduction in terms of the cost and labor intensity of genetic assessment (Bacles and Ennos 2008). In addition, methodological difficulties may arise due to sample contamination (Harrison et al. 2013), in gene typing and statistical probability calculations (Heuertz et al. 2003; Kalinowski et al. 2007). Therefore, alternative approaches focusing on pollen distances per se are needed. Aerobiological methods offer this possibility and provide important information about the distance over which pollen can disperse and potentially pollinate.

Due to the heritability of the disease (McKinney et al. 2011; McKinney et al. 2014; Enderle et al. 2017; Semizer-Cuming et al. 2021), the development of the disease at stand level is highly dependent on the ability of less susceptible genotypes to spread their genes via pollen and seed, both in natural stands and in seed orchards (Lobo et al. 2015; Semizer-Cuming et al. 2019; Semizer-Cuming et al. 2021). Unfortunately, aerobiological methods do not provide information on pollination efficiency and the relationship between pollination success and the health status of ash trees. However, as this relationship has only been studied by Semizer-Cuming et al. (2019), complementary studies on effective pollen transport should be conducted to test whether healthy fathers can make a greater contribution to the next generation.

Furthermore, to date there are no studies that directly examine the extent to which ash dieback affects the quality of male gametophytes. Thus, the disease could alter flower and pollen production as well as pollen quality. In addition to the direct influence on pollen quality, various meteorological influences during the potentially longer pollen transport could also affect pollen viability. Again, this has not been systematically investigated in relation to ash dieback. At the same time, the effects of ash dieback on seed quality should be investigated, as the viability or germination of seeds from diseased ash trees could also be affected. On the other hand, the cultivation and maintenance of less susceptible seedlings could be suitable for genetic conservation and breeding programs, thus providing a measure for the conservation of the threatened tree species (McKinney et al. 2014).

3.2 Objectives and research questions

The research gaps highlight that the risk of reduced genetic connectivity due to progressive fragmentation and the influence of pollen dispersal and pollen/seed quality of differently damaged trees are still largely under-researched. Therefore, the main objective of this thesis was to generate new knowledge on (effective) pollen dispersal as well as on pollen production and pollen and seed quality of ash trees in relation to their health status. For this purpose, this PhD project pursued several sub-objectives:

Firstly, the focus was on ash pollen transport, as ash pollen dispersal has rarely been investigated in aerobiological studies. The influence of phenology, weather conditions and stand density on pollen deposition and transport was considered. Special attention was given to seed orchards in which ash pollen dispersal was studied at a small spatial scale. In this context, (a) the potential impact of increasingly fragmented ash populations on ash pollination and (b) the suitability of the recommended 400 m distance from seed orchards to other ash trees should be assessed. Secondly, we used paternity analyses in a seed orchard and a floodplain forest to test whether there is a relationship between pollination success and health, and to estimate distances of effective pollen transport at different sites affected by ash dieback. Thirdly, this study focused on the effects of health conditions on pollen and seed quality and pollen production at different sites under different environmental/site conditions (e.g., seed orchards vs. natural floodplain forest). Additionally, experiments were conducted to investigate the effects of meteorological factors and long-distance pollen transport on pollen viability.

Specifically, the following main research questions will be addressed in this dissertation:

- (1) How does ash (effective) pollen transport vary with stand density, ash abundance and meteorology?
- (2) Is there a link between mating success and damage to the paternal tree?
- (3) Does the health of the ash trees affect pollen and seed quality, as well as pollen quantity?

3.3 Outline of the dissertation

Following Chapters 1 and 2, which provide an overview of the characteristics of ash trees, their reproductive ecology, and the threat of ash dieback, Chapter 3 presented the unanswered research gaps and the objective of this thesis. The following chapters describe the study sites (Chapter 4) and the data collection methods (Chapter 5). Next, four scientific studies are shown in Chapter 6, which answer the research questions outlined above. In three of the studies, I held first authorship and one study was co-authored with my colleague Lisa Buchner and others (statement of authors' contribution in Chapter 9). In addition, all studies passed a peer-review process and have been published in international scientific journals.

The first publication (Chapter 6.1), "*Aerobiological Pollen Deposition and Transport of *Fraxinus excelsior* L. at a Small Spatial Scale*" (Eisen et al. 2022, Forests), mainly addresses research question (1) and investigated the influence of topography, phenology and meteorology (especially wind) on pollen transport in 2019 and 2020 in two seed orchards in Baden-Württemberg using aerobiological field experiments. We determined the pollen dispersal distance using gravimetric pollen traps and evaluated the recommended distance of seed orchards to other ash trees according to the German Forest Reproduction Act (i.e. 400 m; FOVG 2002; BGBl. I p. 1658).

The second publication (Chapter 6.2) "*Pollination success of Fraxinus excelsior L. in the context of ash dieback*" (Eisen et al. 2023; Annals of Forest Science) refers to research question (1) and (2) and examines the relationship between pollination success and the health status of ash trees, as well as the dispersal patterns of effective pollen transport. We conducted paternity analyses in a seed orchard and a floodplain forest and surveyed surrounding ash trees for mating success, health status and gender. To improve our understanding of gene flow patterns, the locations of mother and father trees were graphically visualized.

The third publication (Chapter 6.3), "*Does ash dieback affect the reproductive ecology of Fraxinus excelsior L.?*" (Eisen et al. 2024; Journal of Forestry Research), addresses research question (3) and focuses on pollen production and pollen and seed viability of ash trees with different health status in two seed orchards and one floodplain forest. The results of this study showed whether ash dieback directly affects the reproductive fitness of ash trees and allowed us to draw conclusions regarding natural selection, the future adaptability and conservation of genetic diversity of ash trees.

The fourth publication (Chapter 6.4), "*Pollen Viability of Fraxinus excelsior in Storage Experiments and Investigations on the Potential Effect of Long-Range Transport*" (Buchner, Eisen [...] et al. 2022; Forests), helps us to answer research questions (1) and (3) and is based on experiments. We investigated storage viability and the influence of meteorological factors during pollen transport on pollen viability. The suitability of four different viability tests was also evaluated.

Chapter 7 provides a summary discussion that aims to explain the main results and place them in the context of previous research. In addition, definite recommendations for gene conservation in forestry practice are presented. Chapter 8 concludes the thesis with a summary.

The summary bibliographies of the Introduction and Discussion are found at the end of the dissertation.

The research presented in this publication-based dissertation was carried out as part of the project "*Quo vadis Pollen? Untersuchungen zur (effektiven) Pollenausbreitung und Pollen- und Samenqualität als Beitrag zur Generhaltung bei der Esche*" (QuoVAPo, P035). The project was financed by the Bavarian State Ministry of Food, Agriculture and Forestry administrated by the Bavarian State Institute of Forestry (LWF) under grant agreement. We worked closely with our cooperation partner Dr. Barbara Fussi, from the Bavarian Office of Forest Genetics (AWG). In addition, a collaboration was established with Dr. Devrim Semizer-Cuming (AWG) and Dr. Branko Šikoparija (BioSense Institute / University of Novi Sad, Serbia).

4 Ash study sites

Two ash seed orchards, *Schorndorf* and *Emmendingen*, in Baden-Württemberg, Germany (hereafter called *Schorndorf* and *Emmendingen* with respect to their locations), and an area of a floodplain forest near *Neuburg an der Donau*, Bavaria, were selected for the studies in this dissertation (Figure 4a-d). These sites were selected because they differ in topography and degree of stocking (Emmendingen - flat and Schorndorf - sloping terrain; floodplain forest - natural forest with dense mixed stocking). The ash trees in the seed orchards are clonal graftings of plus trees that were selected and planted for growth and stem quality prior to the ash dieback outbreak (FVA 2006; Enderle et al. 2015), but there is currently no commercial demand for ash seed. The floodplain forest is a typical natural forest landscape in which the common ash occurs with a share of about 15 % (Jochner-

Oette et al. 2021). In particular, the location of the two seed orchards, with wide-open fields and no obstacles, was considered an advantage for studies of the distance over which most pollen are transported or deposited.

Floodplain forest

The floodplain forest along the Danube between Neuburg and Ingolstadt is located in Bavaria, Germany. The selected area for our intensive monitoring (Figure 1b) is located near the Bergheim barrage (48°44' N, 11°16' E, 375 m a.g.l.) and has an area of about 10 ha. The mean annual temperature is 7.8 °C and the mean annual precipitation is 715 mm (1961-1990) (Schwab et al. 2018). The existing calcareous and nutrient-rich substrate of the site is characterized by former flooding and provides favorable growth conditions especially for *Fraxinus excelsior* (Doben et al. 1996; Margraf 2004). In 2019, 60 ash trees were assessed for vitality and genetics. As a number of ash trees had to be removed to ensure road safety in January 2019, 79 tree stumps that were considered relevant for paternity analysis were also genetically tested. Thinning was also carried out in January 2020 and eight of the 60 ash trees were removed. Therefore, the intensive monitoring area was extended to include eight additional ash trees in July 2020. The height of the studied ash trees varied between 10 and 30 m and the average circumference of the trees was 123 cm.

Emmendingen

The Emmendingen seed orchard (48° 6' N, 7° 52' E, 209 m a.g.l.) is located about 15 km north of Freiburg im Breisgau, between the foothills of the Black Forest and the Kaiserstuhl in the Rhine valley (Figure 4c). There are no significant differences in altitude in the vicinity of the orchard. The mean annual temperature is 10.2 °C and the mean annual precipitation is 882 mm (DWD station Emmendingen-Mundingen, 1981-2010). The predominant soil type is gley, a groundwater influenced soil (LGRB 2021). The orchard was planted in 1995 on former agricultural grassland as an initial afforestation of about 4.3 ha (with boarder planting). The original 228 ash trees were planted on an area of about 2.7 ha with 49 clones, with three to six ramets per clone (FVA 2006). The grafted plus trees originate from the Southern German hill and mountain area (Enderle et al. 2015). Due to the large plot size (7.5 m x 10 m to 15 m x 15 m), no thinning has been carried out so far, in contrast to Schorndorf. Nevertheless, 142 trees had already been removed due to ash dieback, and some crown pruning has been carried out in the course of removal measures (FVA 2006). Since the start of our surveys, there have been 86 ash trees in the seed orchard. In the summer of 2019, 45 (52 %) were classified as healthy and 41 (58 %) as damaged. In 2020, only 33 ash trees (38 %) could be classified as healthy, 52 (60 %) as damaged and one ash died between 2019 and 2020. In July 2021, 84 old ash trees were still alive, of which 37 % were still healthy. The height of the ash trees ranged from 6 to 18 m and the average circumference of the trees was 88 cm. The orchard is bordered by a small stream to the north and east, agricultural land to the east and west, and a residential area to the south. Other ash trees were found at a distance of over 900 m to the northeast of the site.

Schorndorf

The seed orchard Schorndorf (48°46' N, 9° 25' E, 420 m a.g.l.) is located in the Rems valley near Schorndorf, about 20 km east of the city of Stuttgart (Figure 4d). The mean annual temperature is 10.3 °C (DWD station Stuttgart Schnarrenberg, 1981-2010) and the mean annual precipitation is 855 mm (DWD station "Winterbach, Rems-Murr-Kr.", 1981-2010). Geologically, the study area is located in the Keuper, a stratigraphic level of the Germanic Triassic (Geyer and Gwinner 2011). The geological unit on which the orchard is located is the Löwenstein Formation and the predominant soil type is brown earth (Geyer and Gwinner 2011; LGRB 2021). The orchard is located on a NW exposed slope with an inclination of about 6° to 10° and covers an area of about 2.3 ha. It was planted in November 1992 with 68 clones (4-8 ramets per clone) in a 7x7 m planting unit. The original 416 ash trees were planted in 25 rows, with 36 clones consisting of eight female or hermaphrodite

ramets and 32 clones consisting of four male ramets. The clones originated from the hilly and mountainous areas of Southern Germany, as well as from the Alps and the Alpine foothills (Enderle et al. 2015). In the meantime, due to thinning and the negative effects of ash dieback, the majority of the total 416 ash trees (approx. 70 %) have been removed. In 2018, the orchard still contained 123 mature ash trees, of which 58 individuals (47 %) were classified as healthy. In 2019, only 33 % were healthy until 2020, when 28 % of the mature ash trees were classified as healthy, 65 % as damaged and 7 % as dead. In 2021, the number of healthy ash trees stagnated at 28 %, but 62 % were classified as damaged and 10 % as dead. In addition, the orchard consists of 67 stock cuttings that have grown from the graft rootstock but have not yet reached sexual maturity. The height of adult ash trees varies between 3 and 17 m (average 11 m) and the average circumference is 64 cm. The orchard is surrounded by meadows, most of which are bordered by a mixed forest (the Schurwald). The main tree species are beech, spruce and pine. There are no other ash trees in the immediate vicinity (within at least a radius of about 1 km). The orchard itself is mainly covered with ash trees, but there are also occasional cherry trees and, especially at the edge of the orchard, other tree species such as lime, serviceberry and apple.

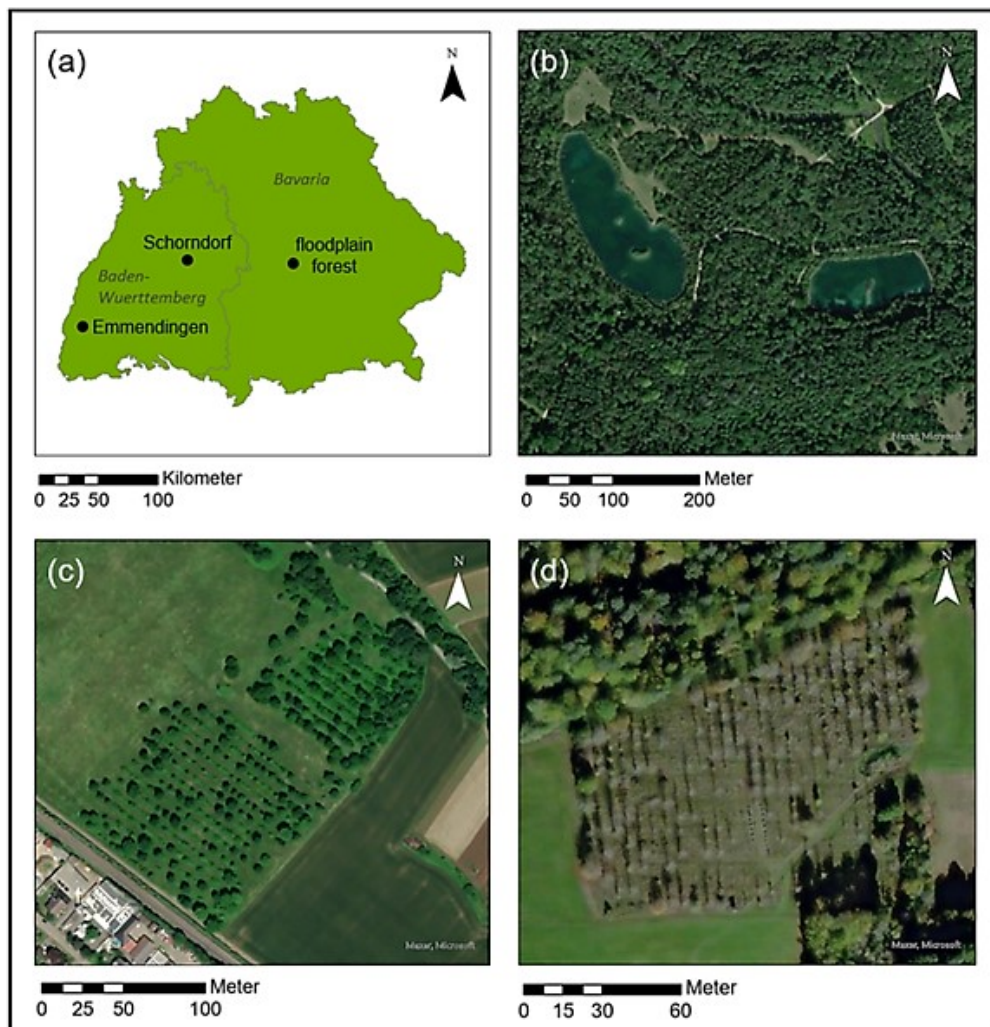


Figure 4: Ash study sites: (a) location of the study sites in Bavaria and Baden-Wuerttemberg, Southern Germany; (b) floodplain forest (48°44' N, 11°16' E, 375 m a.s.l.); (c) seed orchard Emmendingen (48° 7' N, 7° 52' E, 210 m a.s.l.); (d) seed orchard Schorndorf (48°46' N, 9°25' E, 420 m a.s.l.). Source of the maps: ESRI Data & Maps (Eisen et al. 2024).

Additionally, for the fourth publication (Chapter 6.4), we conducted measurements on the roof of the Ingolstadt School of Management (WFI) of the Catholic University of Eichstätt-Ingolstadt, Bavaria (48°76'58.06 "N, 11°41'56.23 "E) at 13 m a. g. l., as there are parks and green areas with numerous ash trees in the surrounding area.

5 Short summary of the applied methods

To answer the research questions, studies were conducted between 2018 and 2022 using different methods:

Vitality assessment

All sampled ash trees were classified annually in July since 2019 (Schorndorf seed orchard since 2018), when maximum leaf mass is available, according to the generally applicable six-level vitality score of Lenz et al. (2012a). Trees in categories 0 and 1 (up to max. 30 % leaf loss) were classified as healthy, trees in category 2 as slightly damaged (up to max. 50 % leaf loss), trees in categories 3 and 4 as severely damaged (up to max. 99 % leaf loss) and trees in category 5 as dead. Other criteria recorded were tree circumference, tree and canopy height, and symptoms of damage such as water sprouts and stem base necrosis, which indicate susceptibility. In addition, the geographic location of all ash trees surveyed was recorded in winter using DGPS (Stonex S9 III, Stonex, Paderno Dugnano MI, Italy).

Phenological observations

Phenological observations were made in spring from early March to mid-May (before and during flowering) according to the BBCH code (Meier 2001). In 2018, the presence of flowers or flower buds and their phenological stage were recorded once in the Schorndorf seed orchard on 19th April 2018 (the 109th day of the year). For 2019 and 2020, phenology was observed at 7-day intervals in both seed orchards and at 3-day intervals in the floodplain forest. Special attention was given to the phenological stages, which represent the onset of flowering and pollen deposition (BBCH 60/61), full flowering (BBCH 65), decrease in flowering intensity and pollen deposition (BBCH 68), and inflorescence fading (BBCH 69). To determine the phenological development stages, the recorded stages were interpolated over the duration of the measurement campaigns, which allowed the estimation of phenological stages between the recorded days. For gender determination of ash trees, the presence of flowers (m/w/z) in spring and seeds in autumn was noted annually.

Meteorological parameters

The meteorological parameters air temperature, precipitation, wind speed and wind direction were obtained from climate stations within the orchards as well as from nearby climate stations of the national German Weather Service (Schorndorf: DWD station Kaisersbach-Cronhütte; Emmendingen: DWD station Freiburg). A weather transmitter (WXT 536, Vaisala, Vantaa, Finland) at a height of two meters including a data logger (1 CR300, Campbell Scientific Ltd., Logan, UT, USA) and solar panels was installed in each seed orchard in March 2019. In addition, two wind anemometers (Skywatch Aero, JDC Electronic SA, Yverdon-les-Bains, Switzerland) were installed in the surrounding agricultural areas in Emmendingen and three data loggers for air temperature and relative humidity (HOBO U23-001, Onset, Southern MA, USA) were installed along the east-west slope in Schorndorf. Cold air flows were modelled using the KLAM_21 software (Sievers 2005; Kossmann and Sievers 2007), since the slope at the Schorndorf orchard (maximum slope 10°) was also believed to have an influence on pollen dispersal (Sofiev et al. 2013).

Aerobiological pollen transport

Aerobiological pollen measurements were carried out with self-constructed gravimetric pollen traps (Jetschni and Jochner-Oette 2021; Wörl et al. 2022) of the Durham type (Durham 1946) in 2019 and 2020. Five Vaseline-coated microscope slides were attached to each trap (one in each direction and one horizontally) to measure cumulative pollen position and pollen transport (Wörl et al. 2022). Due to gravity, airborne pollen deposits on the slides. In the laboratory, the pollen on the slides can be counted using a light microscope (Axio Lab A1, Carl Zeiss AG, Oberkochen, Germany) at 400x magnification (Jetschni and Jochner-Oette 2021) to determine the abundance of pollen (pollen grains/cm²). Slides were changed weekly in both orchards during the ash flowering period (earliest 11th March to latest 14th May). The number of pollen traps per study site varied because the measurement network was adapted to the characteristics and topography of the stands. In Schorndorf, four pollen traps (measuring height: 1.50 m above ground) were set up along a gradient in each of the two years. In Emmendingen, six pollen traps (measuring height: 1.50 m above ground) were set up in 2019 and twelve pollen traps (measuring height: 6 x 1.50 m above ground and 6 x 5.0 m above ground) were set up in 2020.

Paternity analyses (pollination success)

For genotyping, seeds were collected from 12 mother trees each in the Schorndorf ash orchard and in the floodplain forest in October 2018, and cambium samples (i.e., from twigs and stumps) were collected from neighboring ash trees. To quantify the proportion of successful fertilization of diseased and healthy ash trees, DNA was extracted using the cetyltrimethylammonium bromide [CTAB] method (Doyle and Doyle 1990) and genetic analyses were performed using 15 nuclear microsatellites (Brachet et al. 1999; Lefort et al. 1999; Gerard et al. 2006; Aggarwal et al. 2011; Bai et al. 2011; Noakes et al. 2014). The population genetics software GenAlEx 6.0 (Peakall and Smouse 2012) was used for clone identification, and paternity was assigned based on the likelihood model implemented in the Cervus 3.0.7 software (Kalinowski et al. 2007). Paternity distances were calculated using UTM coordinates of mother and father trees.

Pollen production

Pollen production was estimated according to Damialis et al. (2011). For this purpose, four male inflorescences from each direction were collected from 105 ash trees (Schorndorf 2020: N=17; Schorndorf 2021: N=28; Emmendingen 2020: N=24; Emmendingen 2021: N=36) with different health status. In addition, the number of inflorescences per m³ unit crown volume (50 cm x 50 cm x 50 cm; 0.125 m³) was counted to upscale pollen production per total crown volume per tree (Damialis et al. 2011; Ranpal et al. 2022). In the laboratory, the anthers of each inflorescence close to anthesis were soaked in 10 ml of a 10 % potassium hydroxide (KOH) solution (Moore 1999; Fægri 2000) and after 24 h heated and crushed to extract the pollen. Afterwards, 2 mL of safranin solution and 70 % glycerol solution were added to the concentrate to a volume of 20 mL to stain the pollen and prevent clumping (Shivanna and Rangaswamy 1992). 10 µL of the concentrate was placed on a slide and the pollen contained therein was counted using a light microscope (Axio Lab A1, Carl Zeiss AG Axio Lab A1, Carl Zeiss AG, Oberkochen, Germany). This count was repeated twice in order to have a representative average that can be used for pollen count extrapolation.

Pollen viability

To determine pollen viability, pollen from 125 ash trees was collected directly from the study sites (floodplain forest 2019: N=14; Schorndorf 2020: N=23; Schorndorf 2021: N=26; Emmendingen 2020: N=29; Emmendingen 2021: N=33), dusted onto slides, and stained using the TTC test (2,3,5-triphenyltetrazolium chloride) (Stanley and Linskens 1974; Shivanna and Rangaswamy 1992). The test is based on the enzymatic reaction of the dye with living cells that exhibit active respiration (Duro et al. 2013; Castiñeiras et al. 2019). In this reaction, the TTC molecule is reduced by electrons from

the respiratory chain and converted to a red colored substance called formazan. The formation of formazan indicates that the cells are viable and have active metabolic activity. In non-viable or dead cells, the enzymatic activity to reduce TTC is absent and no formazan formation occurs. Therefore, these cells remain colorless and living cells can be distinguished from the unstained dead cells. (Shivanna and Rangaswamy 1992; Krämer et al. 2007). In addition, four other viability tests, such as pollen germination (Shivanna and Rangaswamy 1992), Alexander's stain (Alexander 1969; Peterson et al. 2010), and Acetocarmine (Kearns et al. 1993), were performed in experiments on mixed pollen samples, but were found to be less suitable and practical for determining viability (Buchner et al. 2022).

Pollen storage experiments

The influence of meteorological factors on pollen viability was studied experimentally by storing pollen at different temperatures and humidity for different periods of time and documenting the decrease in viability. For the experiments, a mixed sample of ash pollen from Emmendingen was used. Pollen were collected immediately after the anthers opened and their viability was determined immediately thereafter using the TTC test. The composite sample was then divided and subjected to different storage temperatures (blast freezer: -80 °C; freezer: -20 °C; refrigerator: 4 °C) and the viability of the samples was reassessed after one, two and three months, respectively. In addition, different conditions were simulated in the climate chamber:

- (a) the mean temperature and relative humidity during the main flowering period of ash in April in Bavaria, Germany (day 13 h: 10 °C, 65 % relative humidity; night 11 h: 5 °C, 80 % relative humidity),
- (b) a climate change scenario with a warming of +2 °C, and
- (c) the influence of extreme temperatures (day 13h: 20 °C, 60 % relative humidity; night 11 h: 12 °C, 80 % relative humidity).

Furthermore, during the four-week main ash pollen season of 2021, pollen were collected from the roof of the university building in Ingolstadt, Bavaria using a Burkard volume trap (Hirst 1952) and tested with the TTC-test to investigate the viability of pollen under partially natural transport conditions.

Seed quality

To determine seed quality in relation to ash health status, seeds were collected from 53 ash trees in 2018 and 2020 (2018 (mother trees of paternity analyses): floodplain forest N=12 and Schorndorf: N=18; 2020: Schorndorf: N=15 and Emmendingen: N=8). The seeds were analyzed for quantity and quality (solid grains, hollow grains, insect damage and germination). For this purpose, seeds from 2018 were stratified according to ISTA (2003a) and stored initially for two months at 20 °C and then for seven months at 3 °C in moist quartz sand. Afterwards, the seeds were arranged in the climate chamber at 20-30°C for germination and counted for the first time after 14 days and for the last time after 56 days (Krauss and Köhler 1985). Seeds of 2020 were also tested with the TTC test, as this method provides a faster but equally accurate determination of seed viability (ISTA 2003b; Krämer et al. 2007).

6 Publications

6.1 Aerobiological Pollen Deposition and Transport of *Fraxinus excelsior* L. at a Small Spatial Scale

Article

Aerobiological Pollen Deposition and Transport of *Fraxinus excelsior* L. at a Small Spatial Scale

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Abstract: The ongoing fragmentation of ash populations due to ash dieback requires an effective gene flow between individuals; thus, investigations on ash pollen transport are essential. In this study, comprehensive aerobiological field experiments at two seed plantations in Baden-Württemberg were conducted in 2019 and 2020 in order to study the influence of phenology and meteorology (especially wind) on pollen transport using self-constructed gravimetric pollen traps located 1.5 and 5 m a.g.l.. Our main objectives were to investigate the local scale dispersion of ash pollen and to evaluate the recommended distance (i.e., 400 m) from seed plantations to other ash trees according to the German Forest Reproduction Act. Our results showed a link between pollen transport and meteorology, the onset of phenological development, and the topography of the plantation. The plantation at Schorndorf was characterized by a slope and associated cold air flows, suggesting that this could be a factor contributing to higher pollen levels at the downslope traps. In addition, in many cases, the cardinal direction associated with the highest pollen impaction was also identical with the predominant wind direction. Analyzing pollen data for single traps in detail, we found that the highest total pollen catch (31%) was measured outside the plantations in 2019, a year with very low flower development. In contrast, most pollen (33%) was caught within the plantation in 2020, which presented a much stronger pollen year than 2019 (with a factor of 11 regarding total sums). This indicates, in the lower pollen year, a potential higher contribution of trees from outside the plantation, and thus it can be recommended that seed harvesting of ash trees in the plantations should preferentially take place in full mast years. Interestingly, the total pollen deposition in Emmendingen at 5 m height showed little difference compared to the traps at 1.5 m height, but there was a large temporal difference pointing to vertical variations in pollen availability. In general, we found that ash pollen was transported for a larger distance than 400 m, but the amount of pollen decreased substantially with increasing distance. At a distance of 200 m, there was already approx. 50% less pollen captured from the air. However, even at a distance of 500 m, more than 10% of the pollen was still captured. In order to ensure cross-pollination of healthy ash trees, the distance of ash individuals or stands should not be too large, and there should be no spatial separation (e.g., by conifer stands).

Keywords: ash dieback; seed plantation; gravimetric pollen traps; phenology; meteorology



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1. Introduction

1.1. Ash Dieback—Background

The common ash (*Fraxinus excelsior* L.) has been growing in Europe for centuries [1,2] and is an important and widespread cultivated tree species due to its ecological relevance, climate tolerance, and high-quality wood properties [3–5]. However, during the last decades, its existence has been acutely endangered by ash dieback, which is caused by the invasive fungus *Hymenoscyphus fraxineus* and its asexual stage *Chalara fraxinea*. This fungus

originates from East Asia and has developed an invasive potential in Europe [2,6–8]. Its spores successively attack the leaves, shoots, and wood of the ash trees, which in many cases finally leads to their death [9–11]. Only approx. 1–5% of ashes are believed to be less susceptible [8,12], and a vast decrease in local populations in many forests throughout Europe has already been observed [4,13]. In addition, forestry often reacts to ash dieback with salvage cuts; i.e., increased timber logging or clearcutting [8,14,15]. For example, the proportion in Lithuanian stands, in which ash made up originally between 40–100% only some decades ago, decreased to 0–24% in the following generation due to the large-scale felling of old trees and weak regeneration in the following generation [16]. In Austria, logging increased by a factor of six by 2015 (compared to 2008) [17], and similar increases were recorded for Germany [15] and Denmark [7].

1.2. Consequences of the Ash Population Decline

According to simulations, over 75% of ash populations are expected to decline in the next 30 years in Europe [18], resulting in the fragmentation of ash populations and a weakening of the pollen flow between trees [7]. The common ash is pollinated by wind [2]. Pollen from the father tree reaches the female flower of the mother tree and thereby passes on its genetic information from one generation to the next [19]. This transfer of genes within and between populations influences the genetic diversity of a species as well as the diversity's spatial structure [20]. Thus, individual trees and ash populations are "genetically networked" with each other via pollen dispersal. In a healthy ecosystem, those genotypes that have the highest fitness under the prevailing environmental conditions succeed in the course of the evolutionary adaptation process [19]. Progressive fragmentation is accompanied by a reduction in gene flow and, consequently, a narrowing of the gene pool [7]. Accordingly, the adaptability of ash is negatively affected, and the risk of inbreeding is increased [19–21]. However, for future robust ash populations, it is essential that genetic diversity is sufficiently high, which requires effective gene flow among individuals. Therefore, phenological synchrony and the relative fecundity of the individuals can be regarded as important features [22–24]. However, complex interactions in ecosystems imply that long-term effects of a reduced gene flow are difficult to assess. Due to ongoing fragmentation and the risk of reduced gene flow, studies on ash pollen transport are consequently of mayor importance.

1.3. Seed Plantations

Seed plantations are defined as selectively composed breeding populations used to produce high-quality forest reproductive material [19]. When considering seed plantations, a spatial limitation of pollen flight distances might be desirable. The emission of pollen from other ash trees could have adverse effects on seeds since the source and quality of ash pollen emissions remain unknowns. The German Forest Reproduction Act [25] suggests a minimum distance of 400 m between seed plantations with a low fitness population of the same species to be sufficient to avoid unintended gene flow from other ash populations.

1.4. Previous Studies on Pollen Transport

Information on pollen transport of ash has been mostly gathered indirectly through DNA-based paternity analyses [26–28]. However, distances of the effective pollen transport differ widely among studies; e.g., from 70–140 m [26] to 2900 m [27]. Semizer-Cuming et al. [20] reported that only 5% of ash pollen is transported over longer distances than 1.3 km. However, approaches based on paternity analyses are linked to some drawbacks. In addition to methodological difficulties in gene typing [26], it is challenging or often unfeasible to sample all trees that represent a potential pollen source for reproduction [27]. Due to the reported differences in the estimated pollen transport distances and methodological drawbacks, alternative approaches focusing on pollen flight distances per se using aerobiological methods are needed. While these distances alone are not critical for pollination efficiency, studies of ash pollen transport increase our knowledge and provide

important information on the radius over which the pollen of (susceptible) individuals can spread and potentially pollinate. In general, pollen dispersal and deposition is known to be influenced by abiotic and biotic factors such as meteorological conditions and vegetation, but also tree height and crown width [29–31]. This knowledge is mostly derived from aerobiological studies, and it has also been reported that pollen deposition from single groups of trees outside the forest prevails only over short distances. In a study on the dispersal of *Sequoiadendron giganteum* pollen, Anderson [32] reported that most pollens are deposited only within 100 m of the source. Bricchi et al. [33] studied pollen deposition for a group of 60 *Platanus* trees and showed that about 25% of the pollen was deposited within 400 m of the source, and only nine grains were found at 2750 m. Adams-Groom et al. [30] determined that 94% of pollens from isolated trees of *Carpinus betulus*, *Cedrus atlantica*, *Juglans nigra*, and *Platanus acerifolia* were deposited directly under the canopy.

1.5. Aim of the Study

Since ash pollen dispersal has rarely been investigated through aerobiological studies, we focused on ash pollen deposition and transport, which was assessed using a set of gravimetric traps installed at two seed orchards in Baden-Württemberg in 2019 and 2020. We considered the influence of phenology and weather conditions on pollen quantities. Our main objectives were to investigate the distribution of ash pollen within a small scale in order to assess (a) the possible effects of increasingly fragmented ash populations on ash pollination and (b) the suitability of the recommended distance of 400 m from seed orchards to other ash trees; e.g., characterized by a lower vitality.

2. Materials and Methods

2.1. Study Area

In order to generate generalized knowledge on ash pollen transport, we chose two ash seed plantations, Schorndorf and Emmendingen, in Baden-Württemberg (Germany). These sites were selected since they differ in topography (Emmendingen—flat versus Schorndorf—inclined terrain). In addition, the surrounding area with vast open fields and without obstacles was regarded as an advantage for investigating the distance in which most of the pollen is transported or deposited.

The plantation Schorndorf (48°46′ N, 9°25′ E, 420 m a.s.l.) is located in the valley Remstal near Schorndorf, east of the city of Stuttgart (Figure 1a). Meteorological data were derived from two stations since information on temperature was missing at the closest one. The average annual temperature is 10.3 °C (German Meteorological Service (DWD) station “Stuttgart Schnarrenberg”, 1981–2010 [34]) and the average precipitation sum is 855 mm (DWD station “Winterbach, Rems-Murr-Kr.”, 1981–2010 [34]). The plantation is located on a NW exposed slope with an inclination of about 6° to 10° [35]. It was established in 1992 in a 7 × 7 m seedling cluster and has an area of approx. 2.3 ha. Initially, 68 clones (416 ash trees) were planted in a total of 25 rows: 36 clones with eight female or hermaphroditic individuals and 32 clones with four male individuals. In the past, half of the ash trees were removed due to thinning measures and the adverse effects of ash dieback. The plantation comprised 120 ash trees with a maximum height of 17 m in July 2019 and 2020. According to the scoring system of Lenz et al. [36], 33% were classified as healthy (crown defoliation <30%) and 67% as damaged (crown defoliation 31–100%) in 2019. In 2020, 32 individuals (27%) were classified as healthy and 88 (73%) as damaged. From the 120 ash trees, 51 individuals were identified as female, 54 as male, and 15 as hermaphrodites (Table A1). The plantation is surrounded by meadows, which are mostly bordered by a mixed forest (mainly beech, spruce, and pine), but further ash trees are not present in the immediate surroundings (within a radius of approx. 1 km).

The seed plantation Emmendingen (48°6′ N, 7°52′ E, 209 m a.s.l.) is located approx. 15 km north of Freiburg im Breisgau, between the foothills of the Black Forest and the Kaiserstuhl in the Rhine valley (Figure 1b). There are no noticeable differences in altitude around the plantation. The average annual temperature is 10.2 °C and the average pre-

precipitation sum is 882 mm (DWD station “Emmendingen-Mundingen”, 1981–2010 [34]). The plantation was established in 1995 on former agricultural grassland with an area of approx. 2.7 ha. Originally, 49 clones (228 trees) were planted. In contrast to the Schorndorf seed plantation, thinning measures were not applied so far. Nevertheless, 142 trees already had to be removed due to ash dieback. Since the beginning of our surveys, 86 ash trees (maximum tree height: 18 m) still existed in the seed plantation, of which 45 individuals (52%) were classified as healthy and 41 (58%) as damaged in summer 2019. In 2020, only 33 ash trees (38%) could be classified as healthy. From the 86 ash trees, 17 individuals were recognized as female, 49 as male, and 20 as hermaphrodites. The plantation is bordered in the north and east by a small stream, in the east and west by agricultural land, and in the south by a settlement area. Additional ash trees were detected only in the forested area northeast of pollen trap 6 (Figure 1b).

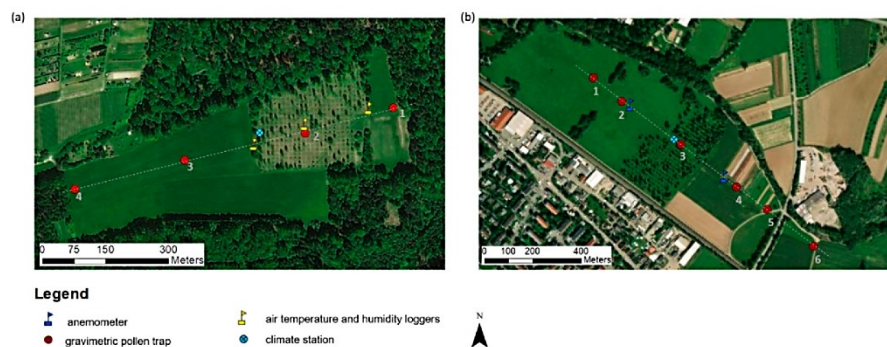


Figure 1. Study areas: (a) seed plantation near Schorndorf ($48^{\circ}46' N$, $9^{\circ}25' E$, 420 m a.s.l.), (b) seed plantation near Emmendingen ($48^{\circ}6' N$, $7^{\circ}52' E$, 209 m a.s.l.) including measurement sites: red circles—gravimetric pollen traps; yellow flags—air temperature and humidity loggers; blue flags—anemometers; blue circle—climate station. Source of the map: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGrid, IGN and the GIS User Community.

2.2. Aerobiological Pollen Measurements

The aerobiological pollen measurements were carried out during the flowering period of ash at the specific sites: Schorndorf: 18 March to 14 May 2019 and 18 March to 13 May 2020; Emmendingen: 18 March to 14 May 2019 and 11 March to 1 May 2020. To adjust the start and end of the pollen measurement campaigns, phenological observations were used.

To measure cumulative weekly pollen deposition and transport, self-constructed gravimetric pollen traps of the Durham type were installed in the selected survey areas [37,38]. These have been designed to attach five Vaseline coated microscope slides (one at each cardinal direction and one horizontally) under a rain shelter (Figure 2).

The measurement network was adapted to the characteristics and topography of the plantations. One gravimetric pollen trap was installed in the center of each ash seed plantation to estimate the quantity of emitted pollen. Horizontal dispersion of pollen was assessed by placing additional gravimetric pollen traps at 1.5 m a.g.l. along dominant wind directions, ensuring that pollen deposition both upwind and downwind from the ash stand was represented. Besides one pollen trap installed directly in the plantation, one was set in the northeast and two in the southwest on the agricultural land surrounding the plantation at a distance of up to 450 m at the seed plantation Schorndorf (Figure 1a). The maximum distance was considered to exceed the distance of 400 m recommended by the German Forest Reproduction Act [25], but was also limited by local conditions (e.g., the proximity of the forest). At the seed plantation Emmendingen, three additional traps were set in the southeast and two in the northwest at a distance of up to 500 m (Figure 1b). In 2020, six additional pollen traps carrying only horizontal microscope slides were installed

above at a height of 5 m a.g.l. in order to better understand vertical pollen distribution. The microscopic slides were replaced on a weekly basis, which allowed for the analysis of the quantity of pollen dispersed around the ash stands with a seven-day temporal resolution.

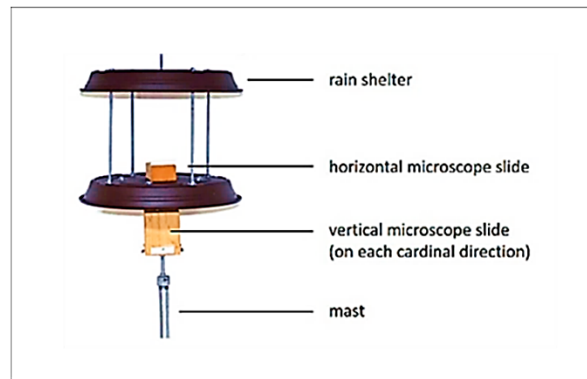


Figure 2. Self-constructed gravimetric pollen trap of the Durham type.

The pollen on the collected microscope slides were stained with a gelatin gelvatol solution that contained safranin and fixed with a coverslip. The prepared slides were then protected with a clear varnish. After curing, the slides were analyzed using a light microscope (Axio Lab A1, Carl Zeiss AG, Oberkochen, Germany) at 400× magnification. Ash pollen was counted along four longitudinal transects, which resulted in the screening of 11.6% of the total slide. Pollen was converted to pollen grains per square centimeter of the impaction surface (pollen grains/cm²).

We analyzed spatial and temporal variations in pollen distribution over each study location. The “weekly pollen catch” (sums of pollen on the horizontal and all four vertical slides per week) was used to analyze characteristics of pollen emission and transport, while the “total pollen catch” (the sum of “weekly pollen catches” for all samplers in the study location) enabled a comparison between seasons regarding the amount of emitted pollen. Furthermore, we analyzed pollen transport/impaction on the vertical slides in relation to the cardinal direction and the intensity of the flowering phase (main pollen season and pre- or post-pollen season). Since there is no standard for defining the flowering season derived from pollen data obtained with gravimetric sampler [38], we visually delineated the main pollen season and defined it as the phase in which more than 50% of the registered pollen catch occurred. In turn, the pre-season was defined as the period before and the post-season as the period after the main pollen season, whereby the phases were limited to account for not more than 25% of the total pollen catch.

2.3. Phenological Observations

Phenological observations prior to and at flowering were conducted to analyze the relationship of flower phenology and pollen deposition. For the years 2019 and 2020, phenology was observed according to the BBCH Code [39] on a weekly basis. Thereby, the phenological stages BBCH 60 and BBCH 61 represent the beginning of flowering and the start of pollen release (maximum 10% of flowers open/release pollen), BBCH 65 represents the full flowering (>50% of flowers release pollen), and BBCH 68 represents the decrease of flowering intensity and pollen release. At stage BBCH 69, flowers were already faded and no longer associated with pollen release. To determine the phenological developmental stages, the weekly recorded stages were interpolated over the duration of the measurement campaign, which allowed the estimation of phenological stages between the recorded days.

2.4. Meteorological Parameters

Meteorological parameters were obtained from the climate stations (Weather Transmitter WXT 536, Vaisala) installed in each ash seed plantation with the aim of determining the influence on pollen deposition and transport. The climate station had a height of two meters and a minimum distance of five meters to the nearest tree. The parameters used for further analyses in this study were air temperature, precipitation, wind speed, and wind direction. In Emmendingen, two anemometers (Skywatch Aero, JDC Electronic SA, Yverdon-les-Bains, Switzerland) were additionally placed in the surrounding agricultural areas, which served for a more detailed investigation of the variation of wind direction and speed. In addition, the regional wind that prevailed during the measurement campaigns was included. The parameters wind speed and wind direction were obtained from climate stations of the DWD [34] (Schorndorf: station “Kaisersbach-Cronhütte”; Emmendingen: station “Freiburg”).

Since the seed plantation near Schorndorf is located on a slope (inclination maximum 10°), we additionally examined cold air flows. Despite their usually low velocities, cold air flows can have a substantial impact on the dispersion of pollen [40]. For this purpose, the two-dimensional mathematical–physical simulation model KLAM_21 developed by the DWD was used [41,42]. The simulation results of KLAM_21 provided spatial and temporal information on wind direction and speed as well as the height of cold air accumulation during radiation weather/cloudless night. Three air temperature and relative humidity data loggers (HOBO U23-001, Onset, Bourne, MA, USA) were installed in order to determine temperature differences; e.g., caused by possible cold air flows.

2.5. Statistical Analyses

All gathered meteorological, phenological and aerobiological data were descriptively analyzed, and their temporal development were presented. Wind rose plots were generated to investigate the pattern of wind direction and associated pollen impaction. All statistical analyses and visualizations were performed in RStudio (version 1.2.1335.0) or Microsoft Excel 2016.

3. Results

3.1. Plantation Schorndorf

3.1.1. Total Pollen Catch in Relation to Phenology, Temperature and Precipitation

In 2019, a total pollen catch (the sum of all horizontal and vertical slides attached at the four installed pollen traps during eight weeks) of 849 pollen grains/cm² was recorded, and in 2020, the value increased to 9391 pollen grains/cm². Thus, 2020 was a much stronger pollen year than 2019 (with a factor of 11) (Figure 3; note the logarithmic scale of the *y*-axis). The maximum amount of pollen was detected in both years around 16 April 2019: 190 pollen grains/cm² (22.4% of the total pollen catch), 2020: 3455 pollen grains/cm² (36.8%). From the beginning of May, only a very small amount of pollen was recorded (2019: 10.0%, 2020: 2.5%). Based on aerobiological data, the pollen seasons were classified as shown in Table 1.

Table 1. Classification of pollen seasons for the seed plantation Schorndorf in 2019 and 2020.

Pollen Season	2019	2020
Pre-season	18 March–2 April	18 March–25 March
Main season	2 April–23 April	25 March–22 April
Post-season	23 April–14 May	22 April–13 May

The temporal pattern of the weekly pollen catch matches well the phenological development of the 69 observed male and hermaphrodite ash trees in the seed plantation. We recorded that only 19 (27.5%) of the male and hermaphrodite trees developed flowers in 2019, compared to 62 (89.9%) in 2020. The height of the ash trees ranged between 3 and 17 m

(average 11 m) high. The average height of the tree trunk was 2.1 m. The inflorescences were located across the entire tree crown.

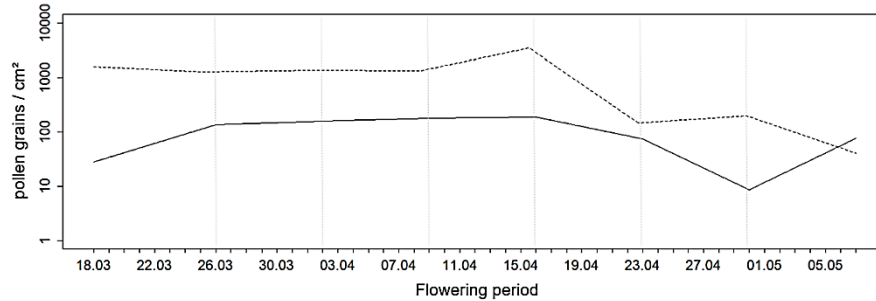


Figure 3. Weekly pollen catch (pollen grains/cm²) presented with a logarithmic scale on the y-axis for horizontal and vertical slides attached at four pollen traps for at the location Schomdorf in 2019 (solid black line) and 2020 (dashed black line).

In 2019, mean March–April temperatures were 8.5 °C. During the measurement campaign, we registered precipitation on 20 days out of 59 (sum: 77 mm, Figure 4a,b). The lowest temperatures (minimum −3 °C) were measured on 20 and 21 March, before the opening of flowers, and did therefore not lead to a falling off of flowers. The onset of flowering and pollen release (BBCH 60 and BBCH 61) was observed on 26 March on three ash trees.

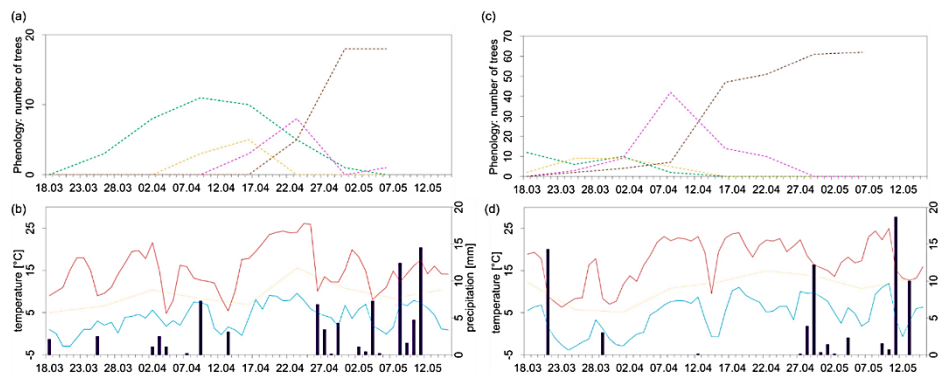


Figure 4. Phenological observations in relation to temperature [°C] and precipitation [mm] during the ash flowering at the location Schomdorf in 2019 (a,b) and 2020 (c,d). (a,c) Phenological observations: BBCH 60 + 61—beginning of pollen release: max. 10% of flowers release pollen (dashed green line); BBCH 65—full flowering: >50% of flowers release pollen (dashed orange line); BBCH 68—end of flowering and pollen release (dashed pink line) and BBCH 69—no more pollen release (dashed brown line). (b,d) daily maximum (red line) and minimum (light blue line), and mean (yellow dotted line) of temperature [°C] and daily precipitation sum [mm] (dark blue bars).

In the pre-pollen season (18 March–2 April), a mean temperature of only 5.8 °C was reached. From 2 April onwards, temperatures rose noticeably and provided the impetus for the start of pollen release for ca. 80% of the ash trees. Full flowering (BBCH 65) started on 9 April and ended on 25 April, which corresponds well with our classification of the pollen season. During full flowering, when also the highest weekly pollen catch was measured, the temperature maximum was reached (24 April: 26.1 °C). Precipitation was especially

high during the post-pollen season (56.8 mm). From 23 April onwards, we recorded the end of flowering and pollen release for most of the trees. Five ash trees were observed that only started to flower at this time (BBCH 60+61). This could explain the small increase in the weekly pollen catch from 29 April onwards. After 30 April, however, only one tree was still in flower.

In 2020, the average March–April temperature was 9 °C (+0.5 °C compared to 2019). The sum of precipitation during the measurement campaign was 68.6 mm (−8.4 mm) on a total of 14 days out of 61 (Figure 4c). Thus, the conditions in the respective periods in spring 2020 were warmer and slightly drier than 2019. Already on 18 March, twelve flowering ash trees in BBCH stage 60/61 and two in stage 65 could be observed (Figure 4d). In addition, temperatures of up to 18.9 °C were measured on this day, which may explain the high weekly pollen catch during this week. On the 21 of March, a total precipitation of 14.3 mm was measured, along with a decrease in temperatures with a minimum value of −3.9 °C on 24 March. This matches the period of stagnation in flower development and could explain the decline in the pollen catch. From 2 April onwards, temperatures started to rise again, and the beginning of flowering and the registered pollen reached its peak. Between 1 April and 8 April, flowering started in most male/hermaphrodite ash trees. During the main pollen season, temperature increased continuously, and there were only 2 days with precipitation. Full flowering (BBCH 65) continued until 16 April. Stage 68 had its peak around 8 April. However, pollen was still released simultaneously from 61 trees around 16 April, even if in varying degrees. This fits well with the maximum of the weekly pollen catch. After 22 April, no more pollen release could be detected, and from 29 April onwards, all flowers had faded. In the post-pollen season, precipitation increased and had a total of 51.2 mm.

3.1.2. Pollen Catch at the Four Traps

For a comprehensive overview, Figure 5 shows the results of the pollen measurements demerged for each pollen trap.

We found that in 2019, the highest total pollen catch was measured at pollen trap 4 (31.4%) and 3 (30.7%), situated 450 m and 225 m to the west of the center of the pollen source, respectively. Trap 2, located within the plantation, had a percentage of 23.1%, and trap 1, at a distance of 150 m to the east of the central point of the plantation, had the smallest percentage of 14.8%. In 2020, most pollen was caught within the plantation (trap 2, 33.4%), closely followed by trap 3 (33.0%). Trap 4 was only associated with 18.8% and trap 1 with 14.7%.

The analysis of pollen that was transported to and impacted on the vertical slides (“pollen impaction”) gives an indication of the wind direction from which the pollen came. Related to meteorological data, the wind direction was predominantly south (30%), southeast (24%), and northwest (15%) in 2019 (wind roses, also for 2020, Figures A1 and A2). Southerly winds were also mostly prevailing when accounting for different pollen seasons (pre-season 36%, main season 31%, post-season 25%). Regarding aerobiological data, most pollen, however, came from the west (35.8%) and east (30.8%). At pollen trap 1, located east of the plantation, the highest pollen impaction was recorded on the microscope slides placed to the west (43.1%), with the second highest in the north (27.6%). These directions fit well with the regional wind (DWD station “Kaisersbach-Cronhütte”), which also was predominately from the west and from the north. Splitting between different seasons revealed that northerly impaction mostly occurred in the post-pollen season. Trap 2 had the highest total pollen impaction in the south (35.5%) and west (31.0%), although a dominance of easterly impaction could be observed in the pre-season. Trap 3 showed the highest impaction in the west (28.7%) and trap 4 in the east (43.5%). In the main pollen season, the dominant direction for both traps was east. In case of trap 3, north was the dominant direction during the post-pollen season. At the furthestmost site (trap 4), almost no pollen was recorded in the pre- and post-pollen season. Thus, our results showed that only at pollen trap 2 did major amounts of pollen impact on the south side of the

trap, which matches with our main wind direction directly measured next to this trap. For all other pollen traps, the dominant wind directions were primarily west and east. Detailed plots showing pollen impactation (also for 2020) can be found in the Appendix A (Figures A3 and A4).

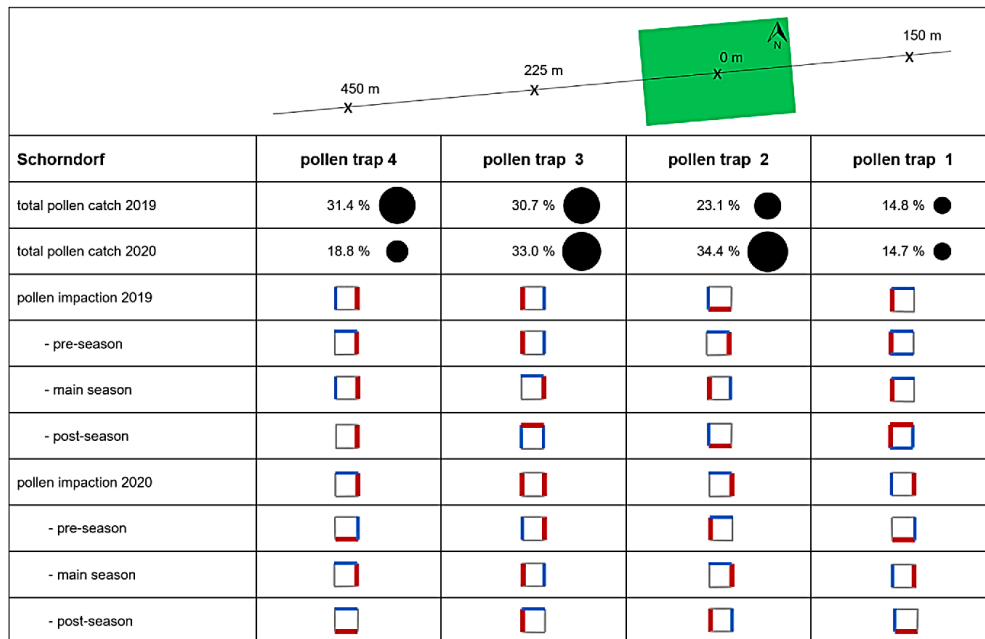


Figure 5. Pollen measurements obtained for the study site Schorndorf. The location of traps (marked with an “x”) and of the plantation (green rectangle) are highlighted at the top of the table. For pollen catch, the size of the circles represents the order of the amount of pollen measured. For pollen impactation, the square represents the pollen trap, the red thick dashes indicate the direction where the most pollen was recorded, and the blue dashes show where the second most pollen was recorded.

In 2020, the dominant wind direction of the plantation was south (34%), southeast (30%), and northwest (10%). During the pre- and main pollen season, it was also south (pre-season 40%, main season 39%). During the post-pollen season, wind was directed mainly from the southeast (30%). Averaged for all gravimetric traps, the most pollen came from east (42.1%) and west (30.3%), which matches well with information on regional winds, which were mainly west, northwest, and northeast. At pollen trap 1, the highest percentage was recorded in the east (46.1%), oriented to the adjacent forest with no ash individuals in the nearer surrounding. The main direction only differed in the post-season, in which it was east. Trap 2 had the highest pollen impactation in the east (37.9%) and north (29.7%). The dominant directions were north for the pre-pollen season, east for the main season, and west for the post-pollen season. Pollen trap 3 was hardly associated with any pollen in the north and south. In contrast, a pollen impactation of approx. 45% each was found in the east and west. For trap 4, oriented towards the plantation, the highest pollen impactation was found in the east (39.2%), followed by north (26.4%). However, during the pre-pollen season, the amount of pollen was very homogeneously distributed across the east, south, and west cardinal directions, and the latter seasons were associated with a primarily northern impactation.

3.1.3. KLAM_21

Another approach to interpreting our aerobiological data was achieved using KLAM_21. Our calculations showed the cold air flows at 2 m a.g.l. that prevail one hour after sunset (Figure 6). Such topoclimatological features can be observed under clear sky conditions and are therefore associated with cold air flows reducing the night-time temperatures at sites located at the lower altitude. Using temperature data measured in the east and west of the plantation (Figure 1a), we also confirmed the existence of lower temperature in the east (2019: 9.5 °C, 2020: 10.7 °C) compared to the west (2019: 9.7 °C, 2020: 10.6 °C).

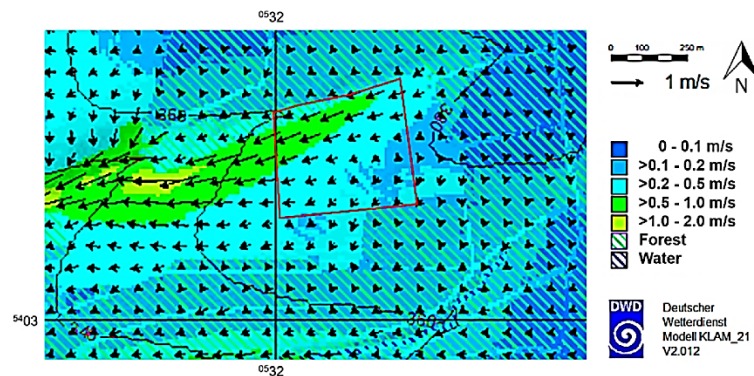


Figure 6. Cold air flows calculated using KLAM_21 (DWD) one hour after sunset. The arrows indicate the magnitude and direction of the flow velocity as an elevation average. The outline of the plantation is marked in red.

3.2. Plantation Emmendingen

3.2.1. Total Pollen Catch in Relation to Phenology, Temperature and Precipitation

In 2019, a total pollen catch of 2647 pollen grains/cm² was recorded during our measurement campaign, and 12,024 pollen grains/cm² were measured in 2020. Again, 2020 was a much stronger pollen year than 2019 (with a factor of 4.5) (Figure 7). However, compared to Schorndorf, the pollen catch in total was consistently higher. In 2020, the pollen season started about one week earlier than in 2019. The maximum amount of pollen was recorded in 2019 between 9 April and 16 April (939 pollen grains/cm² (35.4% of the total pollen)) and in 2020 between 20 March and 27 March (2264 pollen grains/cm² (18.8% of the total pollen)). The main pollen season in 2020 (17 March–17 April) was interrupted between 27 March and 3 April and can thus be divided into two phases. The first phase was from 17 March to 27 March (29.9% of the total pollen catch) and the second phase from 3 April to 17 April (33.8%). From the end of April onwards, only very small amounts of pollen could be registered in both years (2019: 13.8%, 2020: 5.3%). Based on the aerobiological data, the pollen seasons were classified as shown in Table 2.

Table 2. Classification of pollen seasons for the seed plantation Emmendingen in 2019 and 2020.

Pollen Season	2019	2020
Pre-season	18 March–9 April	11 March–17 March
Main season	9 April–23 April	17 March–17 April
Post-season	23 April–14 May	17 April–1 May

The temporal pattern of the cumulative pollen catch also matched well with the phenological development of the 69 observed male and hermaphrodite ash trees in the

plantation. We recorded that 38 (44.2%) of the male or hermaphrodite trees developed flowers in 2019, compared to 58 (67.4%) in 2020. In addition, it was observed that the flowers in 2019 were often not completely developed and were rather stunted. In Emmendingen, the height of the ash trees ranged between 6 and 18 m, with most ash trees varying between 11 and 14 m in height. The average height of the trunk was 2.3 m. Here, the inflorescences were also located over the entire tree crown.

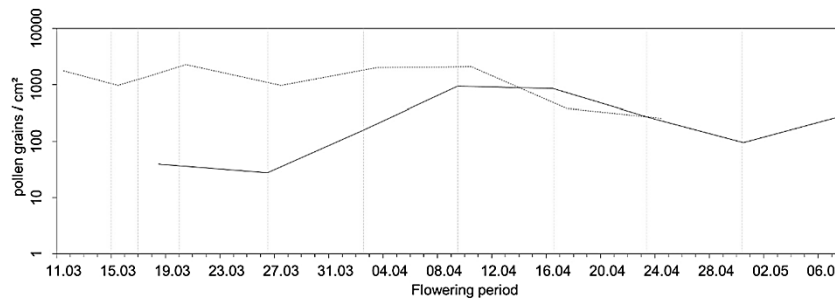


Figure 7. Weekly pollen catch (pollen grains/cm²) presented with a logarithmic scale on the y-axis for horizontal and vertical slides attached at four pollen traps for at the location Emmendingen in 2019 (solid black line) and 2020 (dashed black line).

In 2019, the mean March–April temperature was 10 °C. During the measurement campaign, we registered precipitation on 27 days out of 59 (sum: 125 mm, Figure 8a,b). Thus, this location received substantially more precipitation during the measurement campaign than Schorndorf. As in Schorndorf, the lowest temperatures (minimum −2.6 °C) were measured on the 20 and 21 March. The onset of flowering and pollen release (BBCH 60 + 61) was observed on 26 March on eight ash trees. In the pre-pollen season (18 March–9 April), a mean temperature of 8.3 °C was reached, and temperatures rose constantly until 2 April. However, this was followed by a temperature decrease and substantially more precipitation. The onset of rainy episodes affects the beginning of pollen release, which can be clearly seen in the fluctuations of the curve for stage BBCH 60/61. The first observation of full flowering (BBCH 65) was on the 2 April on six ash trees. This stage reached its peak between 9 April and 23 April, which again corresponds well with our classification of the pollen seasons. The maximum temperature (20 April and 24 April: 25.6 °C) during the measurement campaign was also reached in this period. On 30 April, 87% of the ash trees had reached BBCH stage 68. During the post-pollen season (23 April–14 May) precipitation was especially high (62.4 mm). At the end of the measurement campaign, only one ash tree was still in flower.

In 2020, the average March–April temperature was 11 °C (+1 °C compared to 2019). The sum of precipitation during the measurement campaign was 13.7 mm (−111.3 mm) on a total of 9 days out of 52 (Figure 8d). On the 11 March, two flowering ash trees were observed at BBCH stage 60/61 and one at stage 65 (Figure 8c). At this time, a maximum temperature of 17.2 °C was measured. A week later, on 17 March, 22 ash trees began to release pollen, eight ash trees were in full flower, and the one that was already in full flower on 11 March was in the end of its flowering and pollen release. Between 17 March and 3 April, the beginning of pollen release could be detected in most of the ash trees. The full flowering (BBCH 65) reached its maximum values between 20 March and 17 April. This coincides well with the peak of the pollen catch. Between 20 March and 27 April, 26 ash trees were at stage BBCH 60/61 and 14 at stage BBCH 65. The interruption around 3 April was confirmed both by pollen data and phenology. At this time, the lowest temperatures (31 March: −5.1 °C; 2 April: −3.2 °C) were measured. After that, the temperature increased and there was no precipitation. Between 10 April and 17 April, eight trees were still at

stage BBCH 60/61 and 18 at stage BBCH 65. By 24 April, only two ash trees could still be associated to stage 68. In addition, precipitation resumed from the 26 April onwards. During the post-pollen season, a precipitation sum of 34.1 mm were recorded.

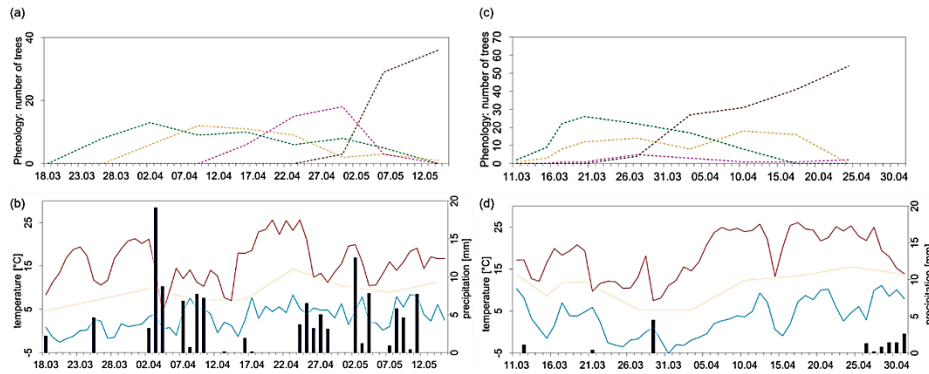


Figure 8. Phenological observations in relation to temperature [°C] and precipitation [mm] during the ash flowering at the location Emmendingen in 2019 (a,b) and 2020 (c,d). (a,c) Phenological observations: BBCH 60 + 61—beginning of pollen release: max. 10% of flowers release pollen (dashed green line); BBCH 65—full flowering: >50% of flowers release pollen (dashed orange line); BBCH 68—end of flowering and pollen release (dashed pink line) and BBCH 69—no more pollen release (dashed brown line). (b,d) daily maximum (red line) and minimum (light blue line), and mean (yellow dotted line) of temperature (°C) and daily precipitation sum [mm] (dark blue bars).

3.2.2. Pollen Catch at the Four Traps

For a comprehensive overview, Figure 9 illustrates the results related to the study site Emmendingen of the pollen measurements demerged for each pollen trap.

Analyzing pollen data for single traps in detail, we found that in 2019, the highest total pollen catch was measured at the two outermost pollen traps, trap 1 (23.1%) and 6 (18.9%), situated 400 m west and 500 m east of the center of the pollen source, respectively. Trap 3, located within the plantation, had a percentage of 15.4%, while trap 5, situated 300 m to the east of the central point of the plantation, had the lowest percentage of 11.6%. Pollen traps 2 and 4, situated 200 m west and east of the pollen source center, had 16.6% and 14.4%, respectively. In contrast, in 2020, most pollen was caught at pollen trap 3 (32.3%), located in the center of the plantation. The lowest pollen catch was found in the most distant pollen traps. Thus, traps 1, 5, and 6 each had only about 12% of the total pollen count.

In 2020, we additionally compared pollen deposition (horizontal slides) for the traps at 1.5 m height and 5.0 m height. It is interesting that the total pollen deposition of all traps placed at 5 m height (5035 pollen grains/cm²) showed only slight differences compared to the traps placed at 1.5 m height (4863 pollen grains/cm²). For both heights, the highest amount of pollen was registered within the plantation (trap 3, 1.5 m: 40.1%; 5.0 m: 41.4%) and the lowest at trap 5 (1.5 m: 9.5%; 5.0 m: 9.3%). However, there was a huge temporal difference. For example, at a height of 1.5 m, trap 3 already showed very high values between 15 March and 17 March (380 pollen grains/cm²), but at a height of 5 m, only 116 pollen grains/cm² were registered. However, this reversed in the period between 17 March and 20 March, where 681 pollen grains/cm² were detected at 5 m height, and only 335 pollen grains/cm² at 1.5 m height. This temporal shift was also obvious when focusing on the peak dates of single traps (Table 3).

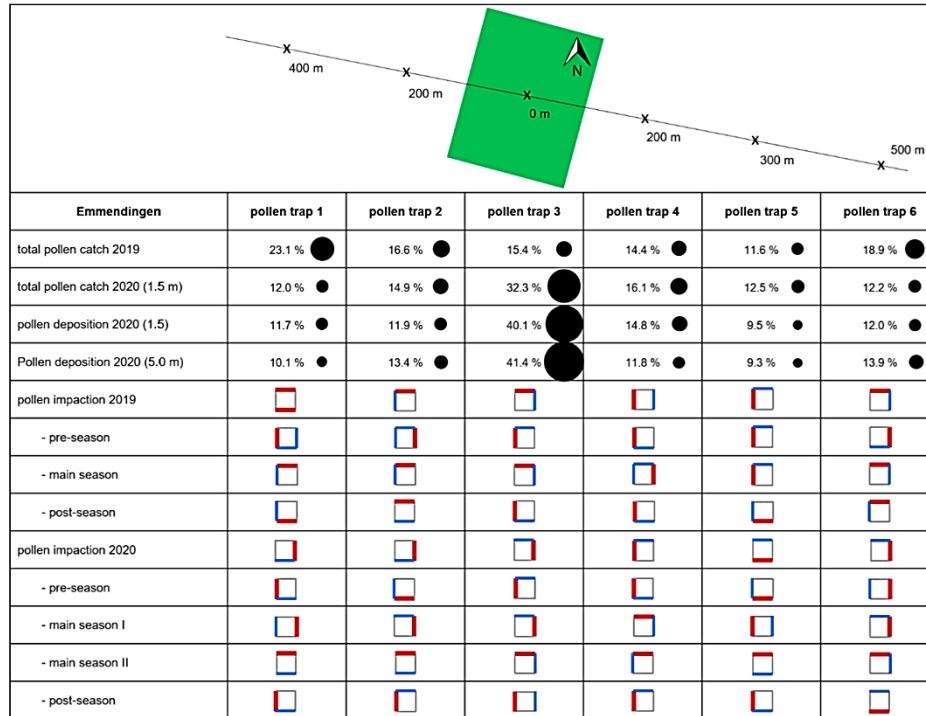


Figure 9. Pollen measurements obtained for the study site Emmendingen. The location of traps (marked with an “x”) and of the plantation (green rectangle) are highlighted at the top of the Table. For pollen catch, the size of the circles represents the order of the amount of pollen measured. For pollen impact, the square represents the pollen trap, the red thick dashes indicate the direction where the most pollen was recorded, and the blue dashes show where the second most was recorded.

Table 3. Peak dates and maximum pollen deposition for single traps at a height of 1.5 m and 5.0 m.

	Trap 1	Trap 2	Trap 3	Trap 4	Trap 5	Trap 6
peak dates 1.5 m	20.03–27.03	11.03–15.03, 10.04–17.04	03.04–10.04	03.04–10.04	10.04–17.04	03.04–10.04
Max. pollen 1.5 m [pollen grains/cm ²]	107	104	424	139	106	139
peak dates 5.0 m	10.04–17.04	03.04–10.04	17.03–20.03	10.04–17.04	10.04–17.04	03.04–10.04
Max. pollen 5.0 m [pollen grains/cm ²]	102	120	681	151	105	149

In Emmendingen, the main wind directions measured at the climate station in the center of the plantation were west (17%), east (16%), and northeast (12%) (Figure A5). The main wind directions recorded by the anemometers located to the west (hereafter Aero1; Figure A6) and east to the plantation (hereafter Aero2; Figure A7) were also west (Aero1: 23%; Aero2 46%). Westerly winds were also mostly prevailing when splitting for different seasons, only during the pre-pollen season, the wind was mainly from the east. Regarding aerobiological data, most pollen came from the north (37.5%) and west (27.5%). At pollen trap 1, located west of the plantation, the highest pollen impactation was found in the north

(31.1%) and in the south (31.3%). Splitting between different seasons, it was obvious that the northern impaction occurred mainly in the main pollen season, and the southern impaction was mainly in the post-pollen season. Trap 2 also had the highest total pollen impaction in the north (54.9%) and the second highest in the west (20.3%), although a dominance of easterly impaction could be observed in the pre-season. Trap 3 in the plantation had the highest pollen impaction in the north (42.5%), followed by east (25.1%). In this case, the predominant direction was north during the main pollen season and west during the pre- and post-pollen seasons. Traps 4 and 5 had the highest pollen impaction to the west, facing the plantation (trap 3: 35.6%; trap 4: 63.0%). For trap 4, west was the main wind direction in the pre- and post-pollen season, and for trap 5, it was west in the pre- and main pollen seasons. For the most distant pollen trap, trap 6, the highest pollen impaction was recorded in the north (51.0%). It is also remarkable that on the western side facing the plantation, only 36 pollen grains/cm² (8.3%) were collected. During the main and post-pollen season, north was the predominant wind direction. During the pre-pollen season, only 3 pollen grains/cm² were registered at pollen trap 6. Detailed plots showing pollen impaction (also for 2020) can be found in the Appendix A (Figures A8 and A9).

In 2020, the dominant wind directions measured at the central climate station were east (18%), northeast (16%), and west (14%) (Figure A10). The main wind directions for Aero1 (west of the plantation; Figure A11) were found to be similar. For Aero2, no data are available for 2020. Based on our data, changes in the direction of local winds occurred more frequently at the plantation in Emmendingen compared to Schorndorf, since the share of main wind directions was considerably lower. This was also indicated by the results of the pollen traps. Averaged for all gravimetric traps, the most pollen came from east (30.5%) and north (27.2%). At trap 1, the highest impaction was recorded in the east (27.9%), facing the plantation. In addition, high values could be registered in the south (26.6%) and west (26.5%). During the pre-pollen season, the dominant direction was south, during the main pollen seasons east and north, and during the post-pollen season west. Trap 2 had the highest pollen impaction in the east (30.7%) and south (26.5%). The dominant directions in different seasons were similar to those of trap 1. Pollen trap 3 showed comparable results to 2019. The highest pollen impaction was registered in the east (42.4%). Again, the dominant side was east in the first main pollen season and north in the second main pollen season. In case of trap 4, the highest pollen impaction was in the west (38.6%), although a dominance of northern impaction could be observed in the main seasons. For trap 5, pollen impaction was highest in the south (36.6%). Split for seasons, the main directions were the same as observed at pollen trap 1 and 2. In the case of trap 6, the highest pollen impaction was observed in the east (39.9%) and north (27.6%). It is also remarkable that on the side facing the plantation in the west, only 11 pollen grains/cm² (13.3%) were collected. The predominant directions during the pre- and first main pollen season were east, the predominant direction during the second main season was north, and the predominant direction during the post-pollen season was south.

4. Discussion

4.1. Relationships between Pollen Catch, Phenology, Temperature, and Precipitation

Our results showed a link between temperature, precipitation, the onset of phenological development, and pollen catch. During the period in which the maximum of pollen was caught, most of the trees were in flower. Thus, the temporal pattern of cumulative pollen catch and our classification of the pollen seasons matched well with our phenological observations, and it can also be assumed that during the period with the highest pollen abundance, mainly local pollen was caught. Thus, phenological observations are a good indicator of pollen dispersion at the local scale. Since in 2019, the onset of pollen release could be observed phenologically on the 26 March at both plantations, it is reasonable to assume that pollen that reached the pollen traps before this date was mainly transported from outside the plantation. Similar results were presented by Estrella et al. [43], who detected that the onset of the birch pollen season in Germany occurred on average six

days earlier than the onset of the local flowering. This discrepancy can be explained by long- or medium-range transport of pollen [43,44]. Such an effect was not noticeable in the stronger pollen year 2020 since pollen was only measured after the first open flower could be observed in the plantation. Although we focused only on two years, our results highlight a sequence of a pollen poor and a pollen rich year. Together with the number of flowering trees and the amount of pollen captured, this points to a resting phase at both plantations in 2019. Gassner et al. [2] noted that there are mast and rest years not only in terms of ash seed production, but also in pollen production. Their pollen analyses showed years with very low pollen concentrations at various sites in Switzerland, followed by years with very high values. Mast years were observed in every second to fourth year, and years with low pollen concentrations occurred synchronously at all study sites in Switzerland. This synchrony coincides with our observations since both seed plantations were associated with a substantial increase of flowers and pollen in 2020.

Average temperature in the months prior to flowering is the most important factor in explaining spring phenological onset dates [44–46]. The rise in temperatures at the end of March 2019 matched well with the start of pollen release, and in the warmer season of 2020, the pollen release started one week earlier than in 2019. The highest pollen catch was measured when temperatures increased above 20 °C. Conversely, it became clear that a drop in temperature is the main reason for a standstill in phenological development, and that this had an immediate impact on pollen catch; for example, during the main pollen season in Emmendingen around 3 April 2020. Temperature is a determining factor for flower growth and development [44,47]. Spatially different temperatures across landscapes which can also occur within small distances [48] can result in a much earlier or later start of the ash pollen season, which could, in case of fragmentation, lead to a failure of pollination as the flowers of the individual ash populations may not mature synchronously.

Precipitation can have an influence on pollen release and concentration [47]. We found that the onset of rain was often linked to temperature decreases, causing a decline in the pollen catch. On the other hand, it was noticed that the onset of rain was associated with an increase in the pollen catch at the end of March in Emmendingen in 2019. In general, the impact of raindrops can also cause a vibration, which lifts pollen inside the flowers or already accumulated on surrounding surfaces such as leaves [49]. Light precipitation during the measurement campaigns had therefore, on the other hand, a minor influence on the weekly pollen catch. High precipitation sums at the end of April 2019 and 2020 were linked to a decrease of captured pollen at both plantations. Thus, rain probably also removed pollen from the atmosphere at the end of the season.

Since in our study, pollen data were only available as a weekly sum, a direct relationship with meteorological conditions, especially precipitation events of a finer temporal resolution, cannot be investigated. Methodologically, it should also be noted that the phenological observations were made only on a weekly basis. In 2019, it was observed that the flowers often did not fully develop and were partially stunted. Therefore, further research should include information on tree specific pollen production. Moreover, an excessive flowering due to an increased damage of trees related to the “forest dieback” in the 1980s was already recognized. In this regard, Gassner et al. [2] suggested that an infection by the invasive fungus *Hymenoscyphus fraxineus* may enhance pollen emission, at least temporarily, as stress symptoms could lead to increased flowering in affected trees. Further research should assess estimations on the pollen production of healthy and affected trees along with an investigation on the viability of ash pollen, which is crucial for pollination success. In addition, a comparison between aerobiological studies and DNA based parental analyses may give important information on the effective gene flow of the endangered ash.

4.2. Estimation of Pollen Transport Distances

Our results showed that the distance over which pollen can be transported depends on various factors. In general, wind is the main factor responsible for the dispersal of pollen [47]. We also revealed a link between wind direction and pollen catch. In many

cases, the cardinal direction associated with the highest possible pollen impaction was also identical with the predominant wind direction. For example, high concentrations of ash pollen in Emmendingen 2020 were transported from easterly and westerly directions, which coincided with the prevailing wind directions. Exceptions were found for example in Schorndorf. These can be explained on the one hand by the formation of cold air flows, and on the other hand by the location of the climate stations (within the plantation) that restricts information of the regional wind. Therefore, we included the climate stations of the DWD (Schorndorf: station “Kaisersbach-Cronhütte”) located in the vicinity and found a better coherence with the prevailing regional wind direction. We demonstrated that the topography of the terrain (i.e., the inclination of the slope) favors the formation of cold air flows, which are able to move pollen following the gradient to pollen trap 4, 450 m away from the center of the plantation. At this measuring site, we found especially high quantities in 2019 (31% of the pollen catch). Pollen trap 1, placed 150 m apart from the plantation, received only very small amounts of pollen in both years. In this case, the wind was hardly directed from the west and mainly from the south and southeast.

In addition, pollen deposition at the Emmendingen plantation, which is located on a flat plain, was also influenced by winds. In 2019, the highest pollen catch was measured at the two outermost pollen traps. Therefore, it cannot be ruled out that pollen from neighboring ash trees might have contributed to higher values on the edge of the study area. This might be especially the case for pollen trap 6, as further ash trees were identified in the forested area northeast of the trap, and the third most common wind direction during the measurement campaign was northeast. Furthermore, the proportion of trapped pollen was less than 10% for traps 4 and 5, located in the east of the plantation. Since there is a dense edge planting on the east side of the plantation, it can be assumed that pollen dispersal is partially blocked. Adams-Groom et al. [30] also found that pollen dispersal within a forest is more limited. In 2020, pollen catch and deposition at the traps located furthest away from the plantation was much lower than compared to the trap within the plantation, so we concluded a considerable decrease in pollen amount with distance. At a distance of 200 m, there was already ca. 50% less pollen deposited. Semizer-Cuming et al. [20], who studied DNA-based pollen transport in ash trees, reported that 50% of ash pollen flew within 140 m and only 5% of pollen farther than 1.3 km. This coincides with our study; however, a finer temporal resolution of the aerobiological data would allow more detailed information on the relationship between pollen and wind.

Another factor was the height of the pollen source in relation to the pollen trap. Pollen traps within the plantations were linked to a lower amount of pollen in 2019 than in 2020. This can be related to the fact that in the weaker pollen year flowers developed mainly in the tops of the tree canopies indicating that pollen was probably carried over the pollen trap. This is also supported by the fact that *Fraxinus* spp. pollen, which has a small diameter of about 20–26 µm and a circular to triangular convex shape [50], can be lifted and transported even at low wind speeds. In order to get closer to the pollen source, additional pollen traps were set up to determine pollen deposition at a height of 5 m in Emmendingen in 2020. We have assumed to measure on average higher quantities at the elevated traps. However, our results showed that the deposition sums did not differ considerably. Although our data only provided weekly averages, we documented substantial temporal differences. Inside the plantation, the peak was reached first at the higher trap; outside the plantation, it was reached at the latest sampling week. Therefore, we conclude that pollen availability at various heights is different and pollination efficiency also varies according to this respective availability. In general, and depending on turbulences, pollen is firstly moved upward from the source and then advectively away with the wind, but begins to sink a few meters from the source [30,51]. This may account for the fact that the highest pollen catch was not registered within the plantation in 2019 at both plantations.

5. Conclusions

The ongoing fragmentation of ash populations will be associated with a decrease in the possibility of effective pollination. This means that the planting density and distance between preferably healthy ash trees are essential and contribute to the exchange of genetic material between trees. Additionally, the results of the study showed a link between pollen transport and meteorology, the onset of phenological development, and the topography of the plantation. For example, we suggest that the slope and associated night-time air movement at the plantation in Schorndorf could be a factor contributing to higher pollen levels at the traps located in the west. In 2019, the low pollen year, the highest total pollen catch was measured outside of plantations. In 2020, the pollen-rich year, the highest total pollen catch was measured within the plantations. This indicates, in the lower pollen year, a potential higher contribution of trees from outside the plantation, and thus it can be recommended that seed harvesting of ash trees in the plantations should preferentially take place in full mast years.

However, to fully understand the dispersion of ash pollen, further studies should consider a finer temporal resolution and more detailed measurements at different heights. Since effective pollination is also dependent on pollen viability, there is a need to incorporate studies on the decrease of viability during long-range transport, which has the potential to pollinate ash trees further away. For this purpose, artificial pollination in different time sequences could be used.

In general, we found that ash pollen was transported over a distance greater than 400 m, but the amount of pollen decreased substantially with increasing distance. At a distance of 200 m, there was already approx. 50% less pollen captured from the air. Nevertheless, even at a distance of 500 m, more than 10% of the pollen was still captured. In order to ensure cross-pollination of healthy ash trees, the distance of ash individuals or stands should not be too large, and there should be no spatial separation (e.g., by conifer stands).

The results obtained from our study are helpful for forestry and seed plantation management and valuable for assessing the future impacts of gene flow in increasingly fragmented ash populations.

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Appendix A

Table A1. Plantation tree information.

		1992/1995	2019	2020
<i>Schorndorf</i>	Number of clones	68	36	36
	Number of ash trees	416	120	120
	Thereof female	288	51	51
	Thereof male	128	54	54
	Thereof hermaphrodite	included in the category "female"	15	15
	Thereof healthy ash trees		33%	27%
	Thereof damaged ash trees		67%	73%
<i>Emmendingen</i>	Number of clones	49	32	32
	Number of ash trees	228	86	86
	Thereof female	NA	17	17
	Thereof male	NA	49	49
	Thereof hermaphrodite	NA	20	20
	Thereof healthy ash trees		52%	38%
	Thereof damaged ash trees		58%	62%

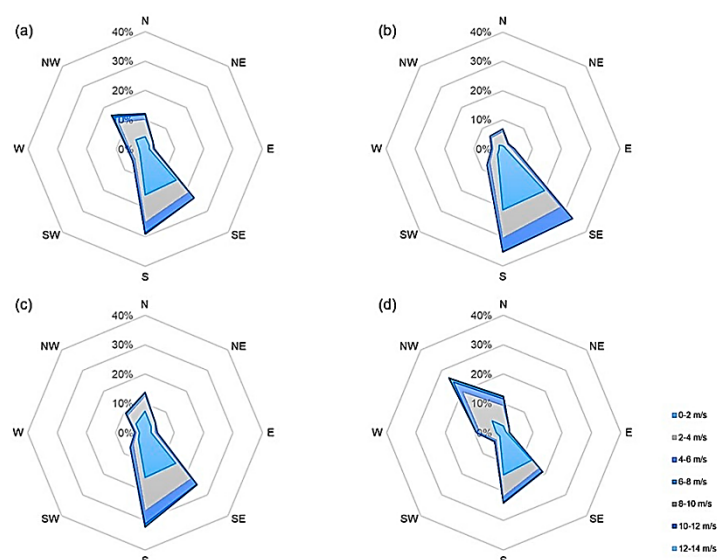


Figure A1. Wind rose during the measurement campaign in 2019 at the location Schorndorf for the periods (a) total: 18.03–14.05, (b) pre-flowering: 18.03–02.04, (c) main flowering: 02.04–23.04, (d) post-flowering: 23.04–14.05.

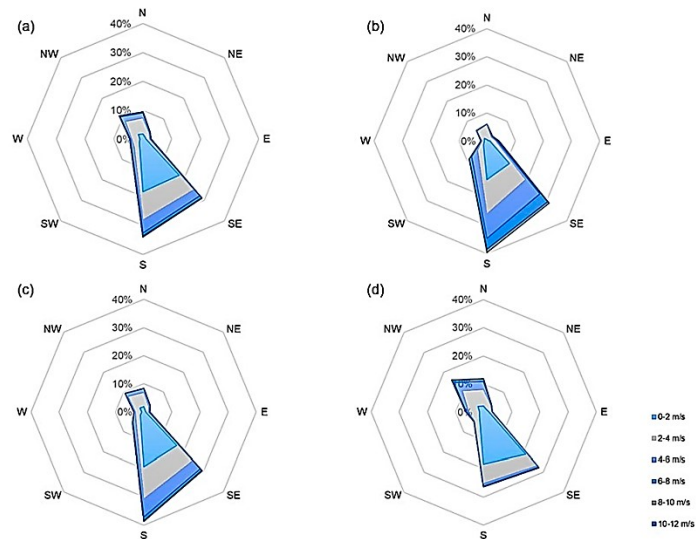


Figure A2. Wind rose during the measurement campaign in 2020 at the location Schorndorf for the periods (a) total: 18.03–13.05, (b) pre-flowering: 18.03–25.03, (c) main flowering: 25.03–22.04, (d) post-flowering: 22.04–13.05.

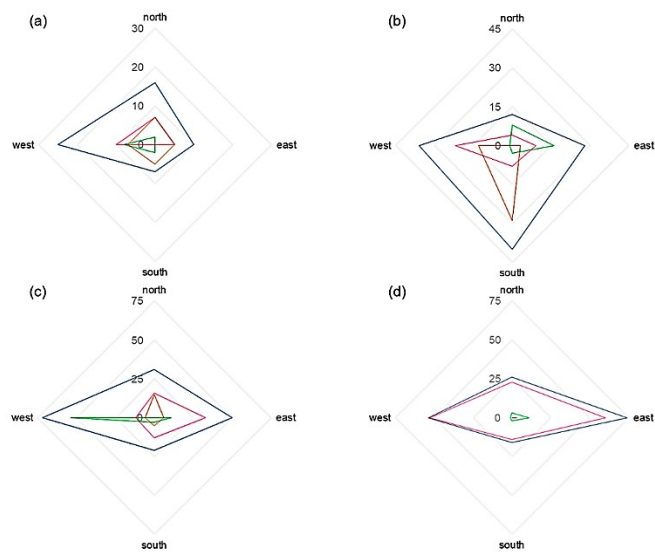


Figure A3. Pollen impact (pollen grains/cm²) on the vertical slides in 2019 for the pollen seasons; pre-pollen season: 18.03–02.04 (green), main pollen season: 02.04–23.04 (pink), post-pollen season: 23.04–14.05 (brown) and total (blue). For pollen traps 1 (a), 2 (b), 3 (c) and 4 (d) at the location Schorndorf.

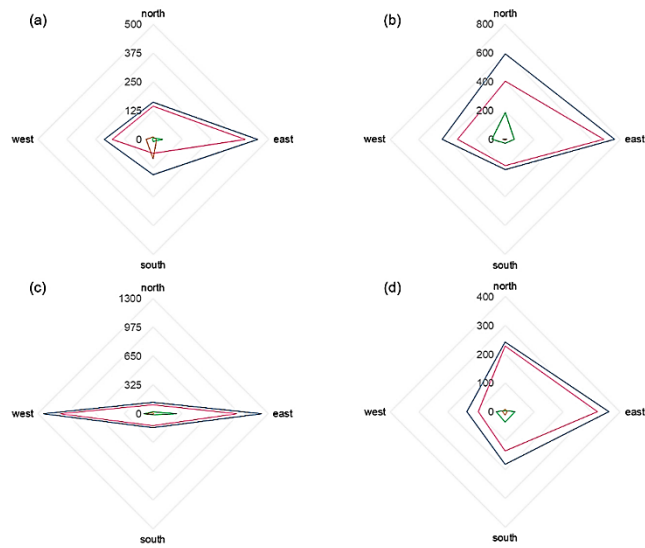


Figure A4. Pollen impaction (pollen grains/cm²) on the vertical slides in 2020 for the pollen seasons: pre-pollen season: 18.03–02.04 (green), main pollen season: 02.04–23.04 (pink), post-pollen season: 23.04–14.05 (brown) and total (blue). For pollen traps 1 (a), 2 (b), 3 (c) and 4 (d) at the location Schorndorf.

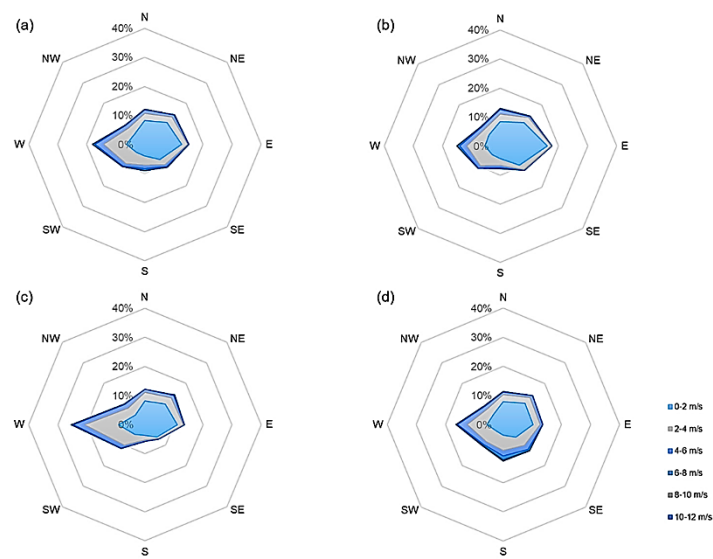


Figure A5. Wind rose from climate station during the measurement campaign in 2019 at the location Emmendingen for the periods (a) total: 18.03–14.05, (b) pre-flowering: 18.03–09.04, (c) main flowering: 09.04–23.04, (d) post-flowering: 23.04–14.05.

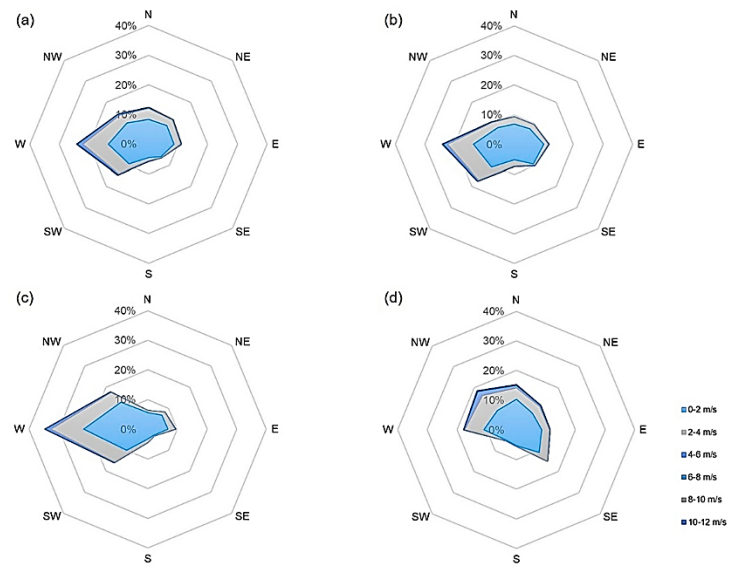


Figure A6. Wind rose from Aero 1 during the measurement campaign in 2019 at the location Emmendingen for the periods (a) total: 18.03–14.05, (b) pre-flowering: 18.03–09.04, (c) main flowering: 09.04–23.04, (d) post-flowering: 23.04–14.05.

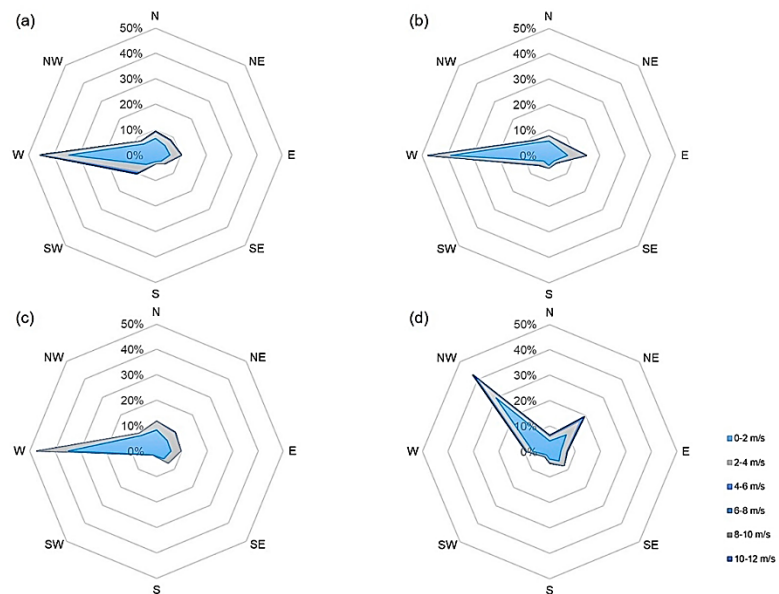


Figure A7. Wind rose from Aero 2 during the measurement campaign in 2019 at the location Emmendingen for the periods (a) total: 18.03–14.05, (b) pre-flowering: 18.03–09.04, (c) main flowering: 09.04–23.04, (d) post-flowering: 23.04–14.05.

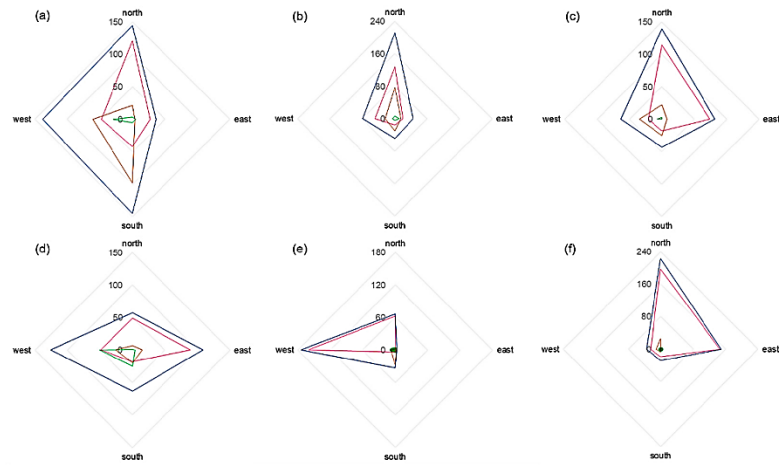


Figure A8. Pollen impactation (pollen grains/cm²) on the vertical slides in 2019 for the pollen seasons: pre-pollen season: 18.03–09.04 (green), main pollen season: 09.04–23.04 (pink), post-pollen season: 23.04–14.05 (brown) and total (blue). For pollen traps 1 (a), 2 (b), 3 (c), 4 (d), 5 (e) and 6 (f) at the location Emmendingen.

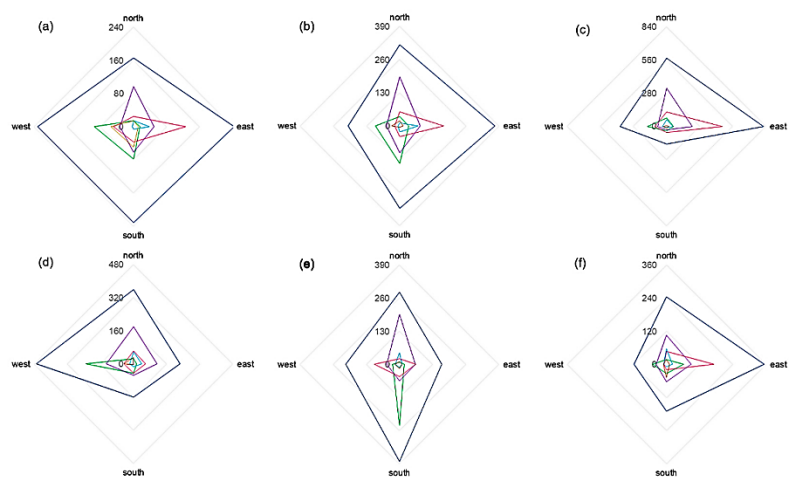


Figure A9. Pollen impactation (pollen grains/cm²) on the vertical slides in 2020 for the pollen seasons: pre-pollen season: 11.03–17.03 (green), first main pollen season: 17.03–27.03 (pink), interruption: 27.03–03.04 (light blue), second main pollen season: 03.04–17.04 (purple), post-pollen season: 17.04–01.05 (brown) and total (dark blue). For pollen traps 1 (a), 2 (b), 3 (c), 4 (d), 5 (e) and 6 (f) at the location Emmendingen.

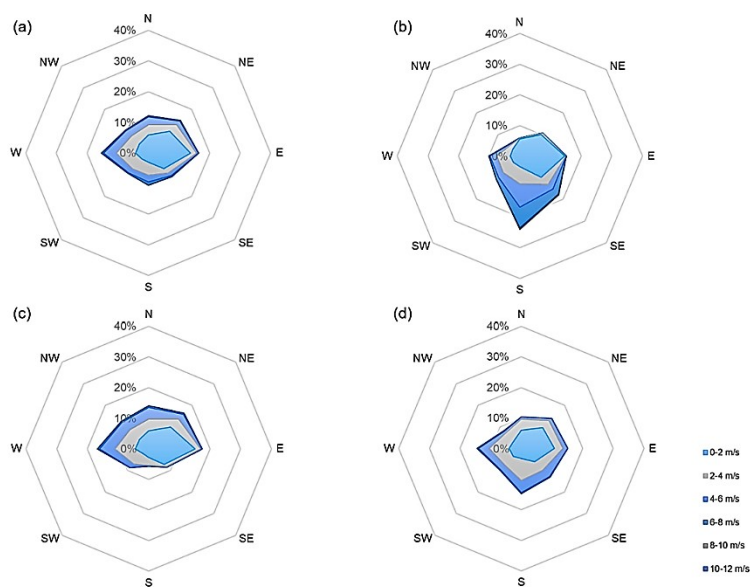


Figure A10. Wind rose from climate station during the measurement campaign in 2020 at the location Emmendingen for the periods (a) total: 11.03–01.05, (b) pre-flowering: 11.03–17.03, (c) main flowering: 17.03–17.04, (d) post-flowering: 17.04–01.05.

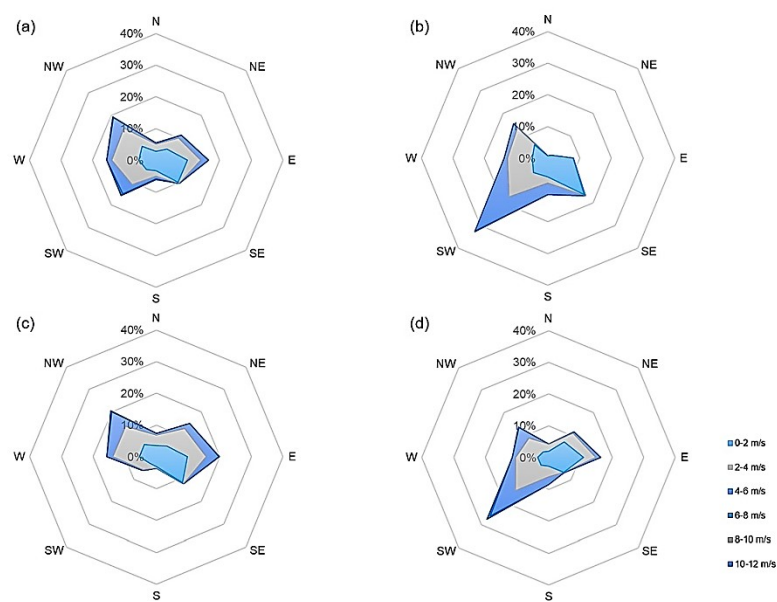


Figure A11. Wind rose from Aero1 during the measurement campaign in 2020 at the location Emmendingen for the periods (a) total: 11.03–01.05, (b) pre-flowering: 11.03–17.03, (c) main flowering: 17.03–17.04, (d) post-flowering: 17.04–01.05.

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6.2 Pollination success of *Fraxinus excelsior* L. in the context of ash dieback

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Pollination success of *Fraxinus excelsior* L. in the context of ash dieback



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Abstract

Key message Paternity analyses show that effective pollination of ash (*Fraxinus excelsior* L.) in a seed orchard and a floodplain forest affected by ash dieback is more likely to be facilitated by healthier males. Thereby, natural selection can have a positive effect on the health of future generations.

Context Ongoing ash dieback and increasing fragmentation of ash populations may result in reduced pollen flow, which can reduce pollination success of future generations of ash trees. Therefore, it is essential to further improve our understanding of gene flow patterns, especially with respect to ash dieback.

Aims In this study, paternity analyses were conducted in a seed orchard and a floodplain forest in Germany in 2018 to explain the relationship between pollination success and the health status of ash trees and distances of effective pollen transport.

Methods Cambium samples (i.e., from twigs and stumps) were collected from 251 ash trees (putative father and mother trees) for genotyping, and the health status of each tree was documented using a scoring system to evaluate vitality. Additionally, seeds were harvested from 12 mother trees per site. Genetic analyses using nuclear microsatellites were performed to determine paternal trees. Paternities were assigned based on the likelihood model implemented in the Cervus 3.0.7 software.

Results Our results showed that the average pollination distance was 76 m in the seed orchard and 166 m in the floodplain forest. In general, pollination success decreased substantially with increasing distance to the mother tree. Despite the dense tree cover in the floodplain forest, pollen were transported over long distances (greater than 550 m), suggesting that non-local sources also play a role in pollination. This is supported by the foreign pollen input identified in the seed orchard (66.5%). Self-pollination was detected only to a very small extent, and thus had no major influence on reproduction. In addition, both healthy and slightly diseased father trees showed similar mating success. However, this was not the case for the severely diseased ash trees (more than 50% of crown damage) because only a few offspring could be assigned to them. Nevertheless, in contrast to the floodplain forest, there was no significant correlation between damage classes and pollination success in the seed orchard.

Conclusion Long-distance pollen transport contributes to the connectivity of ash trees in the landscape. Additionally, both healthy and slightly diseased fathers have a greater contribution to pollination, thus potentially improving the health of the next generation of ash trees. Moreover, gene flow between stepping stone populations is necessary to ensure the positive impact on the genetic diversity of ash populations in the future.

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Keywords Ash dieback, Gene flow, Paternity analysis, Effective pollen transport, Pollination success

1 Introduction

In recent decades, ash dieback, caused by the fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, and Hosoya (Baral et al. 2014) (Syno.: *Hymenoscyphus pseudoalbidus* (Queloz et al. 2011) and its asexual stage *Chalara fraxinea* (Kowalski 2006), has acutely threatened ash populations in Europe (Metzler et al. 2012; McKinney et al. 2014; Kosawang et al. 2018) and its silvicultural future (Enderle 2019). Common ash (*Fraxinus excelsior* L.) is considered a promising tree species under climate change conditions (Kölling 2007; Enderle et al. 2017; LWF 2019; Müller-Kroehling and Schmidt 2019) and presents itself as a versatile and valuable species with high ecological and economical importance (Enderle et al. 2017; Hultberga et al. 2020). However, high mortality rates have already led to a sharp decline in many local ash populations (Lygis et al. 2014; Marçais et al. 2017; Pliūra et al. 2017; Semizer-Cuming et al. 2021). Simulations indicated that up to 75% of ash trees in mixed stands in Europe are expected to die over the next 30 years (Coker et al. 2019). Other studies even suggest that only approx. 1–5% of ash trees are not susceptible and show little or no symptoms related to ash dieback (McKinney et al. 2014; Rigling et al. 2016; Enderle 2019). In this regard, individuals with very low levels of disease symptoms and less than 30% leaf loss are considered partially resistant (i.e., developing less symptoms) and might be suitable for genetic conservation or breeding programs (Lenz et al. 2012; McKinney et al. 2014). Disease development at stand level will therefore depend on the ability of less susceptible genotypes to spread their genes via pollen and seeds (Lobo et al. 2015; Semizer-Cuming et al. 2019, 2021). Partial resistance to ash dieback was not found to be population or provenance based but rather individual based (McKinney et al. 2011, 2014; Enderle et al. 2017; Semizer-Cuming et al. 2019). Therefore, it is essential that genetic connectivity is maintained, even in fragmented landscapes, and that genetic diversity is sufficiently high for the reproduction of trees for future healthy ash populations. To assess tree population responses and the consequences of ecological and biological threats, it is essential to understand pollen dispersal patterns.

Effective gene flow between partially resistant adult trees is necessary to establish healthier next generations of ash trees (Semizer-Cuming et al. 2017, 2019; Fussi 2020). However, ongoing ash mortality and the increasing fragmentation of ash populations lead to a

decrease in pollen flow (McKinney et al. 2014; Semizer-Cuming et al. 2017), which can further limit the genetic diversity of future generations (Fussi et al. 2014). If partially resistant ash trees are highly underrepresented, less healthy genotypes will also succeed and produce offspring. This can lead to decreased natural selection under the prevailing environmental conditions (Eisen et al. 2022b). Reduced genetic diversity can negatively affect the adaptive potential of future generations of ash trees, whereby genetic variation is particularly important for adaptation to new pathogens due to the long generation time of forest trees (Fussi et al. 2014; McKinney et al. 2014). In addition, ash trees are trioecious, i.e., monoecious and dioecious individuals can occur simultaneously (Roloff 1997), which can increase the risk of self-pollination (McKinney et al. 2014; Semizer-Cuming et al. 2021).

Genetic analyses investigating the mating success of ash individuals in populations have been performed only in a few studies to date (Heuertz et al. 2003; Bacles and Ennos 2008; Thomasset et al. 2014; Semizer-Cuming et al. 2017). The relationship between reproductive success and the health status of ash trees in a seed orchard has only been assessed once (Semizer-Cuming et al. 2019): It was demonstrated that health status and reproductive success of ash trees in a seed plantation in Denmark were negatively correlated. Female ash trees severely affected by ash dieback produced a much lower seed quantity compared to healthy ones, whereas damaged males could still sire some offspring. In another study, Semizer-Cuming et al. (2017) examined the genetic connectivity of ash trees in an isolated forest area. Thereby, it was found that about 55–64% of the seeds and 75–98% of the seedlings descended from local ash trees, but 26–45% of the pollen were transported from outside the forest area. Moreover, a positive correlation was found between seed dispersal distance and wind speed, while there was none between pollen dispersal distance and wind speed. Research on distances of effective pollen transport varied widely among studies: Heuertz et al. (2003) conducted a study in a mixed deciduous forest in Romania and determined a distance of 70–140 m, whereas Bacles and Ennos (2008) estimated the distance with up to 2.9 km in a deforested Scottish landscape. Semizer-Cuming et al. (2021) found that 50% of pollen dispersal occurred within 140 m in a Danish forest, 5% of pollen dispersal occurred over a distance of 1.3 km, and 1% was transported farther than 3 km. Similar

results were also obtained from aerobiological investigations on pollen transport and deposition using gravimetric pollen traps, which were conducted in two ash seed orchards in Germany (Eisen et al. 2022a). It was shown that 50% of the ash pollen in the air occurred within a distance of 200 m from the source, whereas only 10% of the total pollen catch reached a distance of 500 m.

In general, pollen dispersal is influenced not only by abiotic and biotic factors such as meteorological conditions, topography, and vegetation but also by tree height and crown size (Scheifinger et al. 2013; Puc 2012; Adams-Groom et al. 2017; Eisen et al. 2022a). Sork and Smouse (2006) also noted that less fragmented landscapes have more pollen input from outside and therefore adjacent pollen sources. The magnitude of influential factors suggests that it is important to improve our understanding of gene flow patterns in fragmented and non-fragmented ash populations further to predict accurate estimates of pollen dispersal (Semizer-Cuming et al. 2017).

Given this background, the objectives of this study are thus to explain the relationship between pollination success and the health status of ash trees and to test whether healthy fathers contribute more to the next generation, as well as to estimate pollen dispersal at sites affected by ash dieback. Based on the results, we discuss the implications of the observed relationship between the extent of damage caused by ash dieback and pollination success in relation to conservation and management of the species. This could be of particular interest for the establishment of future seed orchards where pollen emission from outside should not influence the production of healthy offspring in the orchards.

2 Material and methods

2.1 Study area

The seed orchard Schorndorf (48°46' N, 9°25' E, 420 m a.s.l.) is located in the valley Remstal near Schorndorf, Baden-Württemberg, Germany, and has an area of approx. 2.3 ha (Fig. 1a). The average annual temperature is 10.3 °C (DWD station Stuttgart Schnarrenberg, 1981–2010) and the average annual precipitation 855 mm (DWD station "Winterbach, Rems-Murr-Kr.", 1981–2010). The orchard is located on a NW exposed slope with an inclination of about 6 to 10° (LGRB 2021). It consists of grafts of selected so-called "plus trees," which were selected for growth and stem quality prior to the outbreak of the disease and is characterized by a well-spaced plot design (7 m × 7 m). The plus trees originated from the South German hill and mountain area and the Alps and Alpine foothills in

Baden-Wuerttemberg (Enderle et al. 2014). Initially, 68 clones (416 ash trees) were planted in November 1992 in 25 rows: 36 clones with 8 female or hermaphrodite ramets and 32 clones with 4 male ramets. Due to the negative effects of ash dieback, the majority of the trees (ca. 70%) died (Eisen et al. 2022a). In 2018, the seed orchard consisted of 123 mature ash trees with a maximum height of 17 m (Fig. 1b/Appendix Fig. 7). In the seed orchard, common ash is growing almost exclusively with few other tree species such as cherry and apple trees, wild service trees, or lime trees (especially along the sides). It is surrounded by meadows, which are mostly bordered by a mixed forest consisting mainly of beech, spruce, and pine. There are no other ash trees in the immediate surroundings (within a radius of about 1 km). Due to ash dieback, there is currently no commercial demand for ash seeds.

The floodplain forest is located at the Danube between the cities Neuburg and Ingolstadt, Bavaria, Germany (Fig. 1a). The selected area for our investigations is situated near the Bergheim barrage (48°44' N, 11°16' E, 375 m a.s.l.) and has an area of about 10 ha. The average annual temperature is 7.8 °C, and the average annual precipitation is 715 mm (1961–1990) (Schwab et al. 2018). The calcareous and nutrient-rich substrate of the site enables favorable growing conditions especially for common ash (Doben et al. 1996; Margraf 2004). Common ash is represented in the dense floodplain forest with a share of about 15% (Jochner-Oette et al. 2021). In the selected area, 50 ash trees and 78 tree stumps were sampled for our analyses (Fig. 1c/Appendix Fig. 8).

2.2 Sampling and vitality assessment

In October 2018, a lift truck was used to collect seeds for genotyping in the seed orchard and in the floodplain forest (12 mother trees selected per stand, Table 1) to quantify the proportion of pollination success of diseased and healthy father trees. All investigated ash trees (seed orchard: 123 ash trees and floodplain forest: 50 ash trees) were classified according to the scoring system of Lenz et al. (2012) for assessing the vitality of adult ash trees. Trees in categories 0 and 1 (up to max. 30% leaf loss) were classified as healthy, all other trees as diseased (categories 2 to 4) or dead (category 5). The dead trees were not included in the surveys. In Schorndorf, seeds were collected from nine different clones, representing six healthy and six diseased ash trees. From three clones, two ramets were selected for seed harvest (Table 1). A total of five healthy and seven diseased ash trees were selected in the floodplain forest. The number of analyzed and genotyped seeds per mother tree varied between 48

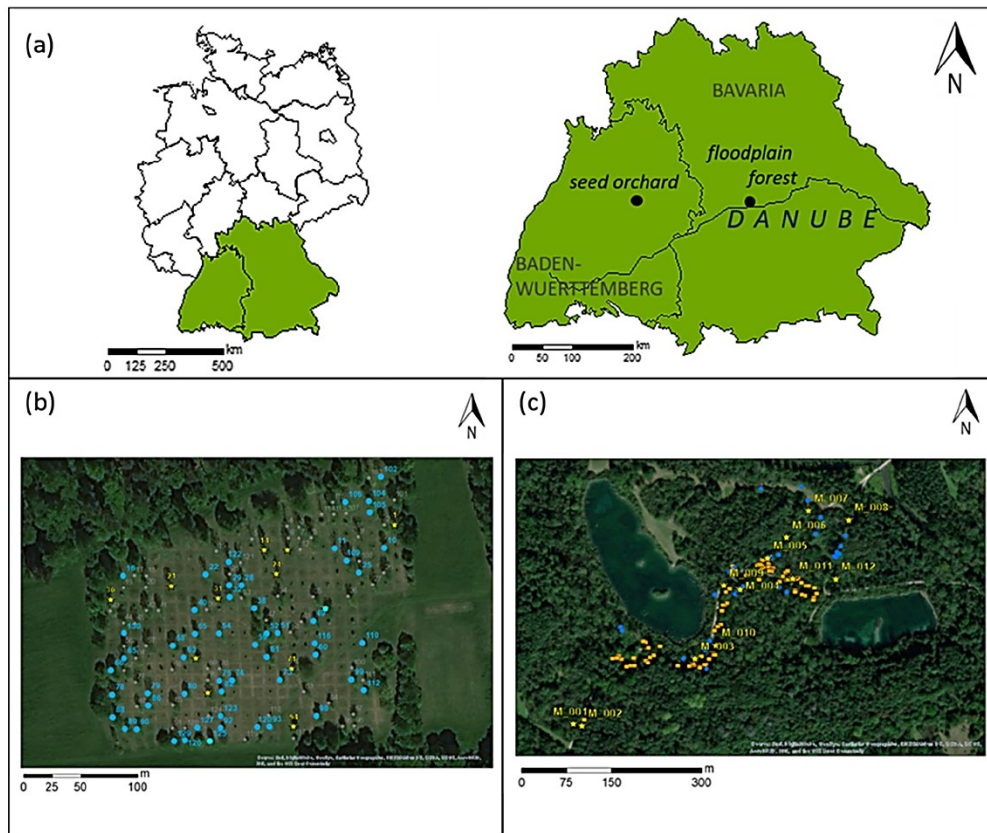


Fig. 1 Study areas. **a** Location of the seed orchard near Schorndorf ($48^{\circ}46' N$, $9^{\circ}25' E$, 420 m a.s.l.) and of floodplain forest ($48^{\circ}44' N$, $11^{\circ}16' E$, 375 m a.s.l.) in Germany. **b** Seed orchard including investigated ash trees, labeled by tree ID; yellow stars — mother trees, blue dots — ash trees identified as potential father trees by their clone number, gray dots — other investigated ash trees excluded as pollen donors. **c** Study area of floodplain forest including investigated ash trees, labeled by tree ID; yellow stars — mother trees, blue dots — ash trees identified as father trees, orange squares — logged ash trees identified as father trees. Source of the maps: ESRI Data & Maps

and 49 seeds in Schorndorf and between 70 and 76 seeds in the floodplain forest.

In spring 2019, twigs from all 123 mature ash trees (12 mother trees and 111 adult ash trees) were sampled in the seed orchard. In the floodplain forest, twigs or wood from 128 trees were sampled: 50 samples were derived from standing ash trees and 78 from ash stumps. The 78 ash trees had to be removed in January 2019 in order to ensure road safety. Since damage caused by ash dieback had already been observed before, they were classified as diseased. The logged trees were ash trees, which may have served as pollen donors since they were cut after

the pollen season and may have contributed to pollination and seed development. The standing ash trees were sampled at a 25-m radius around the mother trees, while ash stumps were interspersed (Fig. 1c/Appendix Fig. 8). After sampling, the tissue was preserved at $-20^{\circ}C$ until further analyses.

Due to the identical genotypes of the clones in the seed orchard, in most cases, a single tree could not be identified as the father (except for the cases where there was only one potential father per clone). Therefore, all ramets of a clone that are possible pollen donors for a mother tree were defined as potential fathers. To exclude the trees that were not candidate fathers (i.e., females and/

Table 1 Characteristics of mother trees and number of analyzed offspring, as well as the potential longest and shortest pollen distance from mother tree to the detected father trees

Study site	Mother tree (clone number _tree ID)	Vitality classification of mother tree in 2018	Gender of mother tree	Number of analyzed offspring	Number of offspring with known father (clone)	Number of (potential) father trees	Shortest distance of pollen transport [m]	Longest distance of pollen transport [m]	
Seed orchard	1619_31	1	Female	48	21	34	6.3	117.8	
	1619_81	1	Female	48	31	17	7.7	111.9	
	1627_01	2	Female	48	10	29	14.7	191.1	
	1627_21	2	Female	48	12	26	27.7	126.9	
	1663_24	2	Female	48	27	24	27.9	124.3	
	1663_94	0	Hermaphrodite	48	9	19	19.9	137.6	
	1664_71	0	Hermaphrodite	49	6	13	14.8	110.8	
	1866_30	2	Female	49	16	26	14.4	171.7	
	0000_62	1	Female	49	29	38	8.2	149.9	
	1878_13	2	Female	48	12	17	39.7	139.1	
	1879_37	1	Female	49	13	25	28.3	135.1	
	1898_14	2	Female	48	5	16	48.1	130.4	
	Floodplain forest	M_01	1	Female	70	13	13	14.8	499.0
		M_02	2	Hermaphrodite	76	10	10	152.0	512.5
M_03		1	Hermaphrodite	70	18	13	19.2	308.8	
M_04		2	Female	73	25	14	3.9	347.2	
M_05		2	Female	71	24	18	5.8	296.0	
M_06		1	Female	72	24	16	32.1	462.7	
M_07		0	Female	70	24	11	2.3	319.9	
M_08		3	Female	71	8	7	40.6	551.6	
M_09		2	Hermaphrodite	74	24	8	24.7	221.1	
M_10		2	Hermaphrodite	76	16	13	16.1	253.5	
M_11		1	Female	70	19	16	5.5	328.2	
M_12		3	Hermaphrodite	74	19	17	0.0	479.8	

or trees with no flowering), the gender of the ash trees was determined on site (in the period 2018–2021), and the presence of flowers or flower buds and their phenological stage was recorded thoroughly with binoculars on 19 April 2018 (109th day of the year). Hermaphrodite mother trees were also considered as potential father trees. For the floodplain forest, no phenological data were available in 2018. The gender was determined for the ash trees alive; however, this was not possible for the ash stumps. The evaluated attributes for all ash trees can be found in the [Appendix](#) (Tables 2 and 3). The geographic position (UTM coordinates; 32 N) of each sampled ash tree was recorded using DGPS (Stonex S9 III, Stonex, Paderno Dugnano (MI), Italy) for both sites ([Appendix Fig. 7/](#)[Fig. 8](#)).

2.3 DNA extraction and genotyping of microsatellite markers

For the adult trees, cambium of the twigs and stumps was used for DNA extraction. For the seeds, DNA was

extracted from their embryos. DNA extraction was performed using the cetyltrimethylammonium bromide [CTAB] method (Doyle and Doyle 1990). The DNA content of the samples was determined using a spectrophotometer (GeneQuant pro, Amersham Biosciences, CA, USA). After DNA extraction, polymerase chain reaction (PCR) was performed to examine 15 microsatellite loci in three different multiplexes ([Appendix Table 4](#)) (Brachet et al. 1999; Lefort et al. 1999; Gerard et al. 2006; Aggarwal et al. 2011; Bai et al. 2011; Noakes et al. 2014). However, reproducible results could only be obtained for eleven markers (M2-30; Fp18437; Femsatl 11; Femsatl 12bis; Femsatl 4; Fp14665; Fp21064; FRESTSSR308; FRESTSSR528; Femsatl 19; ASH2429). Femsatl12bis had too many null alleles in floodplain samples, while the same was the case for marker Fp14665 in the seed orchard. These markers were therefore only used for the seed orchard and floodplain, respectively. Subsequently, PCR products were separated by high-resolution capillary electrophoresis using

the GeXP automated sequencer (Beckman Coulter, Inc., Fullerton, CA, USA) and analyzed by software-assisted allele scoring.

2.4 Data analysis

For clone identification, the population genetic software GenAlEx 6.0 (Peakall and Smouse 2012) was used. The multilocus genotype of all adult trees was compared and identical genotypes assigned to one clone in the seed orchard. Trees with nonidentical multilocus genotypes with any of the known clones were treated as an individual tree and labeled "0000." This was the case when the rootstock had grown up to a new tree (two variants: variant 1: The rootstock has grown up after planting, so the ash trees are sexually mature and could be potential fathers; variant 2: Due to ash dieback, the trees have died, and the rootstocks have sprouted. These ash trees were still too young for flowering). Paternity analysis implemented in the Cervus 3.0.7 software package (Kalinowski et al. 2007) was applied to determine the father of each seed. In this context, the allocation is based on Delta (Δ) (Labuschagne et al. 2015). Thereby, the difference of the likelihood-odds ratio (LOD) score between the two most likely parents is examined to find the actual parents. The critical values of Δ were calculated at strict (95%) and relaxed (80%) confidence levels during the simulations (Labuschagne et al. 2015; Semizer-Cuming et al. 2019). For Schorndorf, 100,000 offspring and 70% of sampled potential fathers were simulated. For the alluvial forest, 100,000 offspring and 30% of sampled potential fathers were simulated. The minimum number of loci was set to eight. The error rate was kept at 0.01 (Semizer-Cuming et al. 2019). In addition, self-pollination was explicitly taken into account in the model.

Pollen distances were calculated from the UTM coordinates of the mother and father trees. In the case of the seed orchard, the distances to the mother trees were calculated individually for all potential fathers (ramets) of the same clone, assuming that each of them could be the potential father, since it was not possible to determine the distinct one. Then, we averaged the sum of the distances for each father tree (i.e., each clone). Thus, it was only possible to estimate the average pollination distances to the mother trees. In the floodplain forest,

we were able to calculate the actual pollination distances between fathers and mothers, as all had unique genotypes. The distances were classified at intervals of 10 m. In addition, the relationship between the number of offspring per father tree and the degree of damage to the father trees were calculated. Since ramets of the same clone were showing varying vitality scores, the average vitality score over all ramets per clone was calculated for the seed orchard Schorndorf. In order to estimate the correlation between the damage class and the pollination success, a chi-squared test and exact Fisher test were applied. All analyses and visualizations were performed in RStudio (version 1.2.1335.0) or Microsoft Excel 2016.

3 Results

3.1 Seed orchard

3.1.1 Paternity analysis: relationship between reproductive success and health status

In total, 123 ash trees could be classified into 37 different clones with distinct genotypes. The number of ramets per clone varied from one to eight, with an average of four ramets per clone in the orchard. Twelve of the trees were selected as mother trees. Of the remaining 111 ash trees, 56 were male, 36 were female, and 15 were hermaphrodites. The gender of four ash trees could not be determined accurately. Seventy-six trees had flowers or flowering buds. Thus, 49 trees (36 females and 13 males/hermaphrodites without flowers) were excluded as pollen donors (Appendix Table 2).

Out of the 580 examined offspring, 194 (33.5%) of them could be assigned to their potential fathers (Fig. 2a). Five single trees and 18 clones (54 ash trees) were detected as potential fathers, although for four clones only one tree could be the father in each case with certainty, due to the exclusion procedure (ash trees that were female and/or not flowering were excluded as fathers). Due to its hermaphroditism and the fact that paternity could be found for this clone, one mother tree was also included as a potential father tree. Nine ash trees had no pollination success; three of them have not reached their reproductive age. Regarding the other six ash trees, four were hermaphrodite, and two were male. It was striking that the two male ash trees were very strongly affected by ash dieback (vitality scores 3 and 4).

(See figure on next page.)

Fig. 2 Results of the seed orchard near Schorndorf. **a** Identified father clones with number of ramets (n) resp. identified fathers as well as average vitality score per clone with the average number of offspring per father tree, **b** number of offspring summed allocated to the average vitality score of the father clones, with n representing the number of genotypes, and **c** average number of offspring summed per potential father tree allocated to the vitality score of the potential father trees, with n representing the number of ramets (vitality score: 0 — dark green; 1 — light green; 2 — yellow; 3 — orange; 4 — red). **d** Number of father trees per distance class based on paternity analysis. All potential father trees per mother tree have been counted

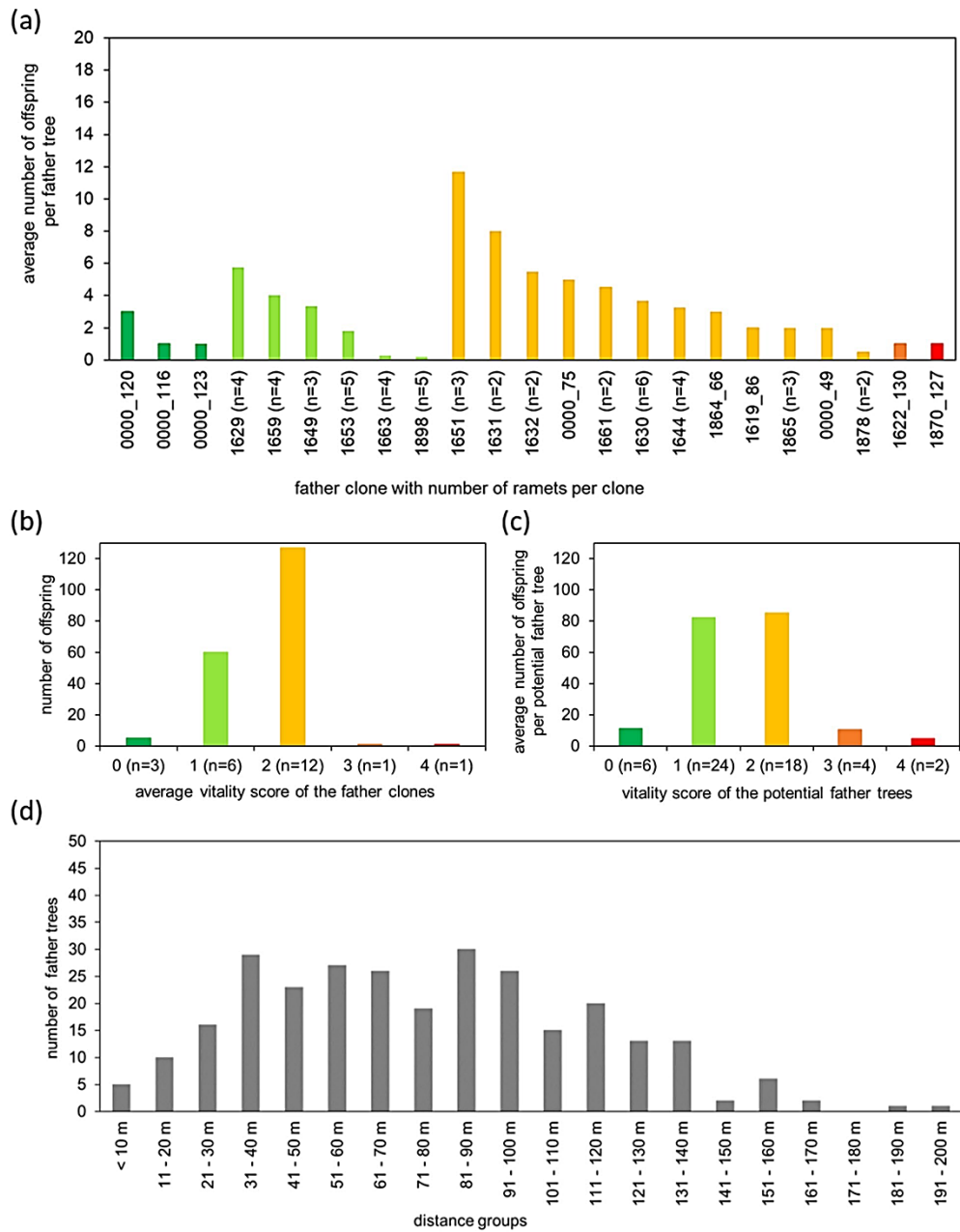


Fig. 2 (See legend on previous page.)

The highest pollination success was observed for the clone *1651* (three potential fathers; average vitality score of 2) with 35 offspring, whereas the lowest pollination success was one offspring associated with 13 trees. Dividing the number of offspring per clone by the number of ramets, the maximum number of offspring is 11.7, and the minimum is 0.3. In addition, the average vitality score over all ramets of a father clone was analyzed; out of the 18 father clones and five single trees, three single trees could be assigned to an average vitality score of 1, and ten clones to an average vitality score of 2. Only one clone each that sired offspring could be assigned to the vitality classes of 3 and 4 (one offspring each). Overall, father trees in vitality class 2 produced the most offspring (127 offspring) (Fig. 2b). In vitality score classes 0 and 1, in which the ash trees are still considered healthy, the number of identified fathers who sired offspring was substantially below the value of class 2 (vitality score 0: 5 offspring and vitality score 1: 60 offspring). However, no significant correlation could be detected between the damage classes and pollination success (chi-square test: $p=0.999$, Fisher test: $p=0.997$).

Considering the 54 potential father trees as individual trees (regardless of the clone affiliation), 6, 24, 18, 4, and 2 ash trees could be assigned to vitality scores 0, 1, 2, 3, and 4, respectively. With an average of 85.6 offspring, most of the father trees were detected in vitality score class 2 in this case as well. However, the number of fathers that sired offspring in scoring classes 0 and 1 was in total higher (vitality score 0: 11.1 offspring and vitality score 1: 82.0 offspring) than the number of offspring in scoring class 2, with an average of 93.1 offspring. Vitality scores 3 and 4 could be assigned an average of 10.6 and 4.7 offspring from identified fathers, respectively (Fig. 2c). Since vitality classes 1 and 2 had more ramets per clone, it can be assumed that weakly damaged clones have a higher overall potential to produce offspring. However, no significant correlation could be found (chi-square test: $p=0.982$, Fisher test: $p=0.904$).

For the mothers, the average number of offspring that could be assigned to a father tree was 7.5 offspring for vitality score 0 (two mother trees), 23.5 offspring for vitality score 1 (four mother trees), and 13.7 offspring for vitality score 2 (six mother trees).

The analyses identified possible self-pollination for three offspring of the mother trees *1619_31* ($n=2$) and *1898_14* ($n=1$). In the clone *1619* ($n=8$), only the ramet *1619_86* was observed to produce male flowers in addition to female flowers. Thus, due to the number of ramets, it can be assumed that there is a

low probability (11.1%) of self-pollination of the parent tree *1619_31*. Clone 1898 consists of two female and six male ash trees; thus, the probability of self-pollination is again very low, with a value of 12.5%. No self-pollination could be detected for the two hermaphrodite mothers.

3.1.2 Distances of effective pollen transport

The average distance of effective pollen transport of all identified potential father trees to the mother trees was 76.2 m in the seed orchard Schorndorf (Fig. 2d). The largest distance was estimated at 191.1 m and could be detected between the potential father tree *1630_90* and the mother tree *1627_1*. The shortest distance was calculated between the potential father tree *1644_32* and the mother tree *1619_31* at 6.3 m.

In general, it was found that only 5.3% of the identified paternal trees with potential pollination success were located less than 20 m from the mother tree. A total of 23.9% of the potential father trees were located between 21 and 50 m from the mother tree, and 45.1% of the potential father trees were located within a distance of 51 to 100 m. For the distance between 101 and 150 m, potential pollination success was attributed to 22.2% of the father trees and for the distance between 151 and 191.1 m only to 3.5%.

For a better spatial understanding of effective pollen transport, pollination success was investigated with respect to the geographical location of the mother trees. The two mother trees, *1619_81* and *1898_14*, located in different areas of the seed orchard were selected as a representative to describe the general observed pollen dispersal patterns.

For mother tree *1619_81* (vitality score 1), most of the offspring (31 out of 48 seeds) could be assigned to their potential fathers (17 different trees) (Table 1). The mother tree is located in the southwestern area of the orchard, and thus, many potential father trees are within reach (Fig. 3a). The results showed that clone *1629* (four ash trees, average vitality score 1) had the highest pollination success (14 offspring) with this mother tree, but with a distance between 54.6 and 111.9 m, it is not located in the immediate vicinity of the mother tree. At the same time, it was found that the father clones with only one offspring are partly very close to the mother tree (clone *1651* — shortest distance of 34.9 m and clone *1879_75* — distance of 10.5 m). Both clones were linked to an average vitality score of 2.

The mother tree *1898_14* (vitality score 2) had the fewest offspring assigned to it (5 out of 48 seeds). However, for the five offspring, 16 possible father trees must be considered with only a maximum of one to two

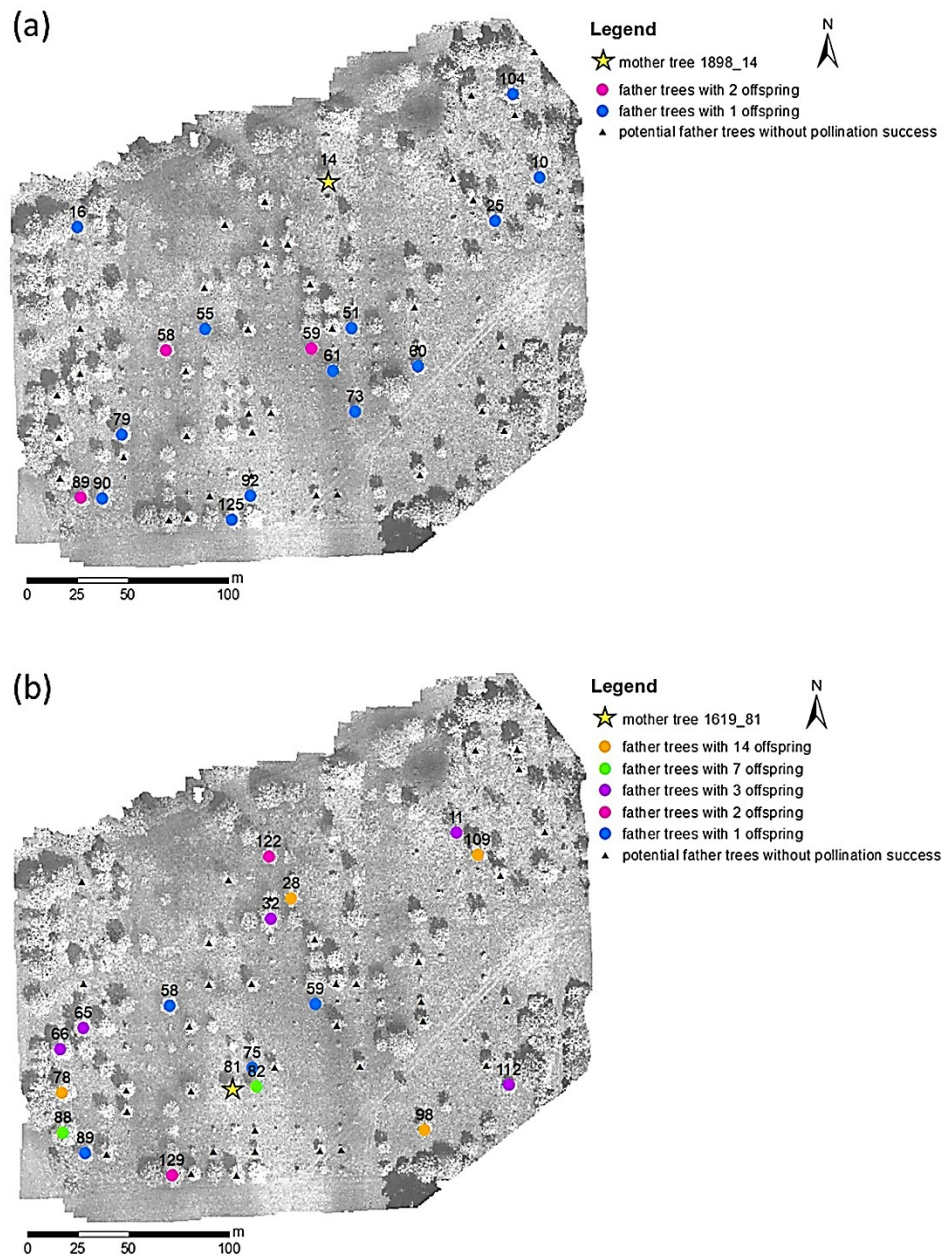


Fig. 3 Mother tree. **a** 1619_81 and **b** 1898_14 with their potential identified father trees and their number of offspring in the seed orchard Schorndorf. Orthophoto taken with drone XR6 (Airborne Robotics, London, UK) equipped with a four-channel multispectral camera (Tetracam Micro-MCA 4, Chatsworth, CA, USA) on 12.07.2018; coordinate system UTM 32N

offspring descended from one father clone. The reason for this could be the location of the mother tree at the northern border of the orchard (Fig. 3b), since 43 offspring could not be assigned to a father clone. The potential father trees identified are not located directly around the mother tree but are mostly distributed in the southern part of the orchard. The closest potential father tree (*1630_51*; vitality score 3) was located at a distance of 48.1 m from the mother tree, 51.6% of the potential father trees were located between 51 and 100 m from the mother tree, and 44.8% of the potential father trees were located between 101 and 130 m from the mother tree.

3.2 Floodplain forest

3.2.1 Paternity analysis: relationship between reproductive success and health status

In the floodplain forest, all sampled trees had unique genotypes. In total, 224 (26.14%) offspring out of 857 examined could be assigned to 73 fathers (Fig. 4 a, b). Forty-three tree stumps and 30 ash trees were detected as father trees. From the 30 living ash trees, 2, 10, 9, 8, and 1 individuals had a vitality score of 0, 1, 2, 3, and 4, respectively (Appendix Table 3). Five of the living ash trees were hermaphroditic; the other 25 were male. Accordingly, no pollination success was detected for 20 living ash trees (out of 50 initially investigated trees), with only six of them being male and one being hermaphroditic; the other 13 were female. Please note that the cambium samples were collected prior to gender determination. Of these seven ash trees (male and hermaphroditic) without pollination success, two were healthy, and five were diseased.

The highest pollination success was observed related to the father tree *tree stump* 19 with 24 offspring, followed by the father tree *A_22* with 20 offspring and the father tree *A_01* with 13 offspring. Twenty-five trees only sired one offspring each. The highest total number of offspring was found in diseased ash trees (vitality scores 2–4). Thus, a significant correlation between the damage classes and pollination success could be found in this case (chi-square test: $p=0.001$). The tree stumps, which were regarded as diseased individuals ($n=43$), had 113 offspring (50.5%), and the diseased ash trees ($n=18$) had 47 offspring (21.0%). In vitality scoring classes 0 and 1, in which the ash trees are

considered as healthy ($n=12$), the number of offspring was 64 (Fig. 4c). In addition, the average number of offspring per number of ash trees in a vitality scoring class was analyzed; it can be recognized that with an average of 12 offspring, father trees of vitality score 0 could be assigned to the most offspring. The father trees of vitality scoring classes 1, 2, 3, and 4 ($n=1$) had an average of four, three, and two offspring, respectively, and the current tree stumps had an average of three offspring (Fig. 4d).

For the mothers, the average number of offspring that could be assigned to a father tree was 24, 18.5, 19.8, and 13.5 offspring for vitality score 0 ($n=1$), 1 ($n=4$), 2 ($n=5$), and 3 ($n=2$), respectively. Additionally, the analyses detected self-pollination for one offspring of the mother tree *M_12* (hermaphroditic; vitality score class 3).

3.2.2 Distances of effective pollen transport

In the floodplain forest, the average distance of effective pollen transport was 165.6 m (Fig. 4e). The longest distance was 551.6 m and could be observed between the *tree stump* 79 and the mother tree *M_08*. The shortest distance (excluding inbreeding) with 2.3 m was calculated between the father tree *A_02* and the mother tree *M_07*.

Overall, 9.6% of the father trees with pollination success were located less than 20 m from the parent tree, and 27.6% were located between 21 and 100 m away. Thus, it can be assumed that the nearest father trees did not pollinate most of the seeds. However, 49.3% of the father trees with pollination success were located within 130 m of the mother tree. A total of 33.3% of pollination success was detected for the trees at more than 200-m distance. For the distance between 301 and 400 m and 401 to 500 m, pollination success could only be detected for 6.4% of the father trees each. For the distance of 501 to 600 m, the value decreased to only 1.3%.

Detailed spatial information was acquired for two selected mother trees, i.e., mother tree *M_04* and *M_08*. For mother tree *M_04* (vitality score 2), 25 seeds out of 73 were attributed to their fathers. This was the mother tree with the highest number of seeds where the respective fathers could be identified. Fourteen father trees

(See figure on next page.)

Fig. 4 Results of the floodplain forest. **a** and **b** Identified **a** living adult trees and **b** father tree stumps (ts) with their vitality score with the number of offspring per father tree. **c** Number of offspring summed allocated to the vitality score of the father trees (n) and **(d)** average number of offspring summed allocated to the vitality score of the number of father trees (n) (vitality score: 0 — dark green; 1 — light green; 2 — yellow; 3 — orange; 4 — red; tree stumps — brown). **e** Distribution of distance classes derived from paternity analyses for pollen dispersal. The distance classes are in relation to the position of the pollen donor (number of father trees grouped)

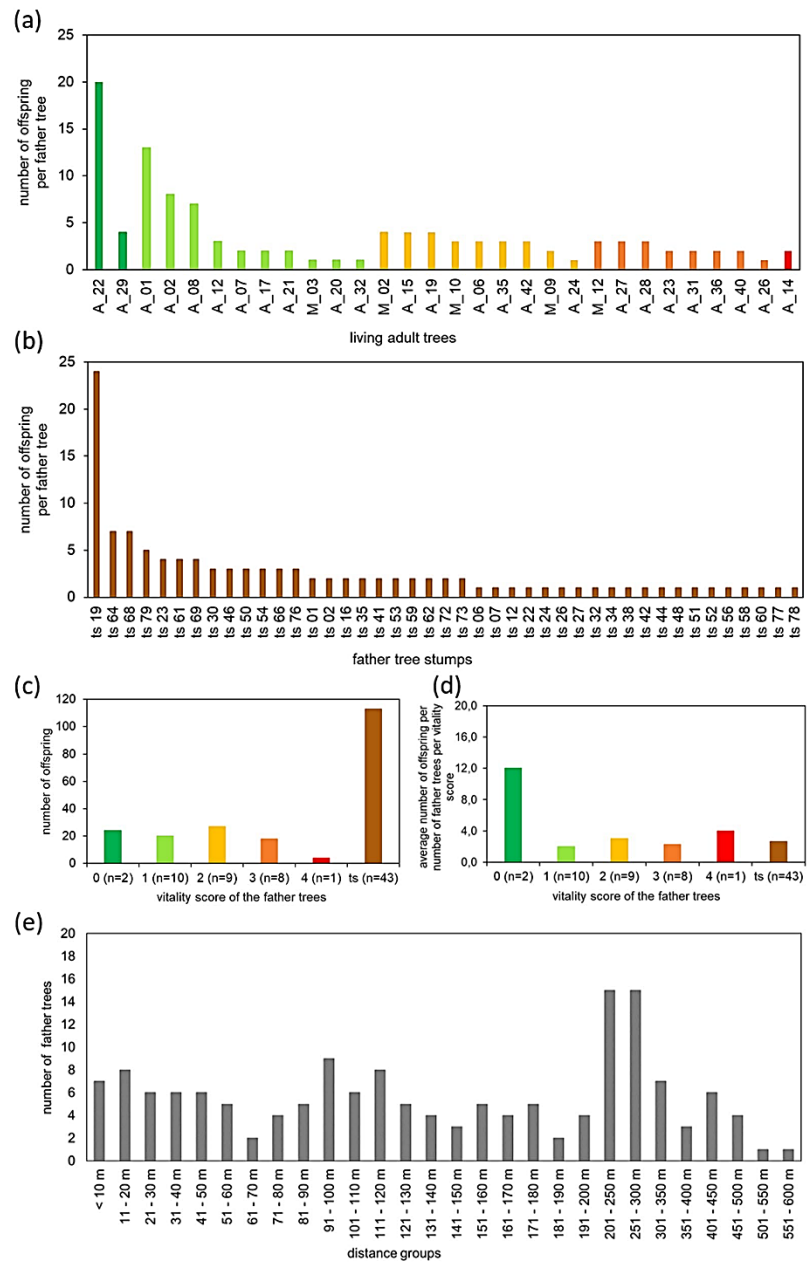


Fig. 4 (See legend on previous page.)

sired 25 seeds (Table 1). The mother tree was centrally located at the study site in a clearing northeast of the lake and was only obscured by other trees to the east and south due to its location (Fig. 5a). Therefore, many of the father trees investigated were within reach to the mother tree. The findings indicated that this mother tree had the highest pollination success (six offspring) with the tree stumps 64 and 68. Both father trees were located on the southwest side of the lake and were not in close proximity, with the distances of 179.9 and 196.8 m, respectively. In contrast, the father ash trees, which were not more than 100 m away from the mother tree, could only be associated with one offspring. In other words, the nearest fathers were not the most successful fathers.

Compared to *M_04*, the mother tree *M_08* (vitality score 3) had less offspring assigned to their fathers. Seven father trees could be detected for the eight offspring. The mother tree is located at the northern edge of the study area; therefore, all identified fathers were positioned in the southwest of the mother tree (Fig. 5b). The closest father (*A_06*) with the pollination success of two offspring was located 40.6 m away from the mother tree. All other more distant father trees had only one offspring. The parent trees, *tree stump 61* and *tree stump 79*, were located the farthest from the mother tree, at a distance of 374.3 and 551.6 m, respectively.

3.3 Comparative summary of the two study sites

The comparison of the results obtained from the seed orchard and the floodplain forest showed that the proportion of all investigated healthy ash trees (vitality scores 0 and 1) differed, with the orchard having 49% healthy trees (Fig. 6a) and the floodplain forest having only 15% (Fig. 6c) or 38% if excluding the tree stumps (Fig. 6e). The results were similar in terms of the proportion of ash trees that could be identified as (potential) fathers (Fig. 6b, d). In this case, the proportion of healthy ash trees was 50% in the seed orchard and 17% in the floodplain forest. However, considering the number of offspring, the analyses showed that at both sites, healthy fathers (vitality scores 0 and 1) as well as those not severely affected by ash dieback (vitality score 2) had almost the same mating success. In contrast, highly diseased fathers (vitality scores 3 and 4), whose proportion in the seed orchard was 14% and in the floodplain forest 30% (excluding the tree stumps; Fig. 6f), could only be assigned to a few offspring. Nevertheless, effective gene flow also takes place between highly susceptible trees.

Although the seed orchard offers the potential to capture most potential father trees, the proportion

of offspring whose father could be identified was not noticeably lower in the floodplain forest either (orchard: 33.5%; floodplain forest: 26.1%). Our results on genetic connectivity showed that the average distance between identified father and mother trees was 76 m in the seed plantation and 166 m in the floodplain forest. Pollination success generally decreased substantially with increasing distance to the mother tree. Despite the dense tree coverage in the floodplain forest, pollen were transported over long distances (greater than 550 m). Thus, it indicates that local sources were not the only ones playing a role in the pollination. This is supported by the 66.5% of foreign pollen input found in the seed orchard. Nevertheless, we were able to identify the differences in pollen dispersal patterns, especially in the seed orchard. For instance, for mother trees at the border of the plantation (*1627_01*, *1878_13*, *1898_14*, *1627_21*, *1866_30*, *1663_94*), only an average of 22% of the offspring could be assigned to their potential fathers, whereas the proportion for mother trees close to the center (*1663_24*, *1619_31*, *1879_37*, *0000_62*, *1664_71*, *1619_81*) was 44%. A similar pattern was also found in the floodplain forest, though it was not as pronounced (near the center: 30%; at the edge: 21%).

4 Discussion

4.1 Relationship between reproductive success and health status

Overall, our results showed that the ash trees of vitality score 2 produced the most offspring on average, both in the seed orchard and in the floodplain forest (excluding tree stumps). But, taking into account the number of individuals, healthy trees with no or little damage (vitality scores 0 and 1) were not associated with substantially lower pollination success. However, it should be noted that in the case of the Schorndorf seed orchard, only the average vitality score class of the clones could be considered for our assessments in the paternity analyses. Due to the identical genotype of the ramets per clone, the software cannot detect a distinct father tree. As the ramets per clone can have varying vitality scores, the average vitality score has been used. Therefore, it is possible that healthy ash trees will produce more offspring than diseased trees in a clone or vice versa. In the floodplain forest, some of the father trees were only present as tree stumps due to the tree removal before sampling. Therefore, we cannot accurately estimate their vitality score but assume that they were damaged by the fungus to some extent (vitality score > 2). Thus, based on the results, both healthy and relatively

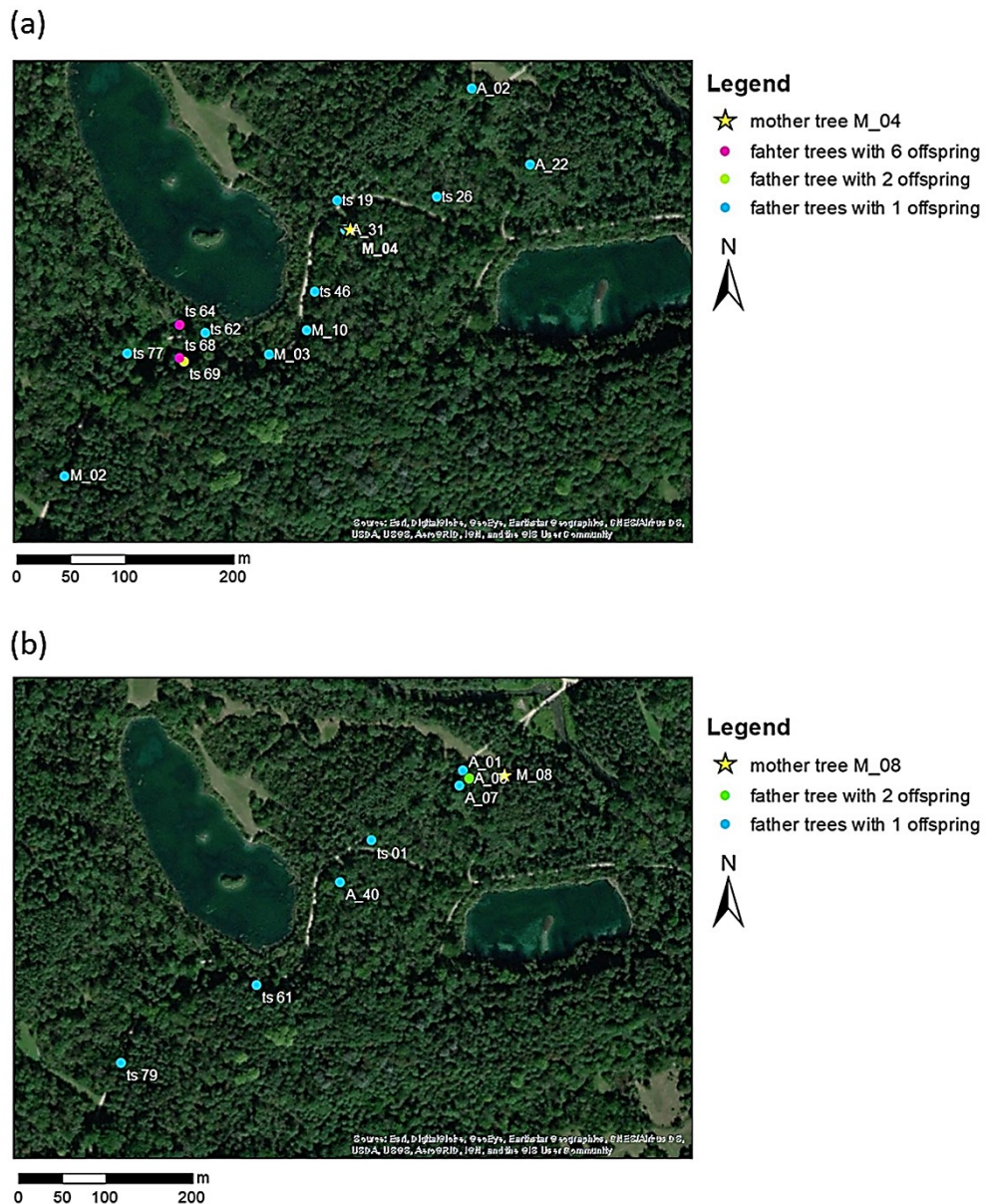


Fig. 5 Mother tree **a** M_004 and mother tree **b** M_008 with their identified father trees and their number of offspring in the floodplain forest. Source of the maps: ESRI Data & Maps

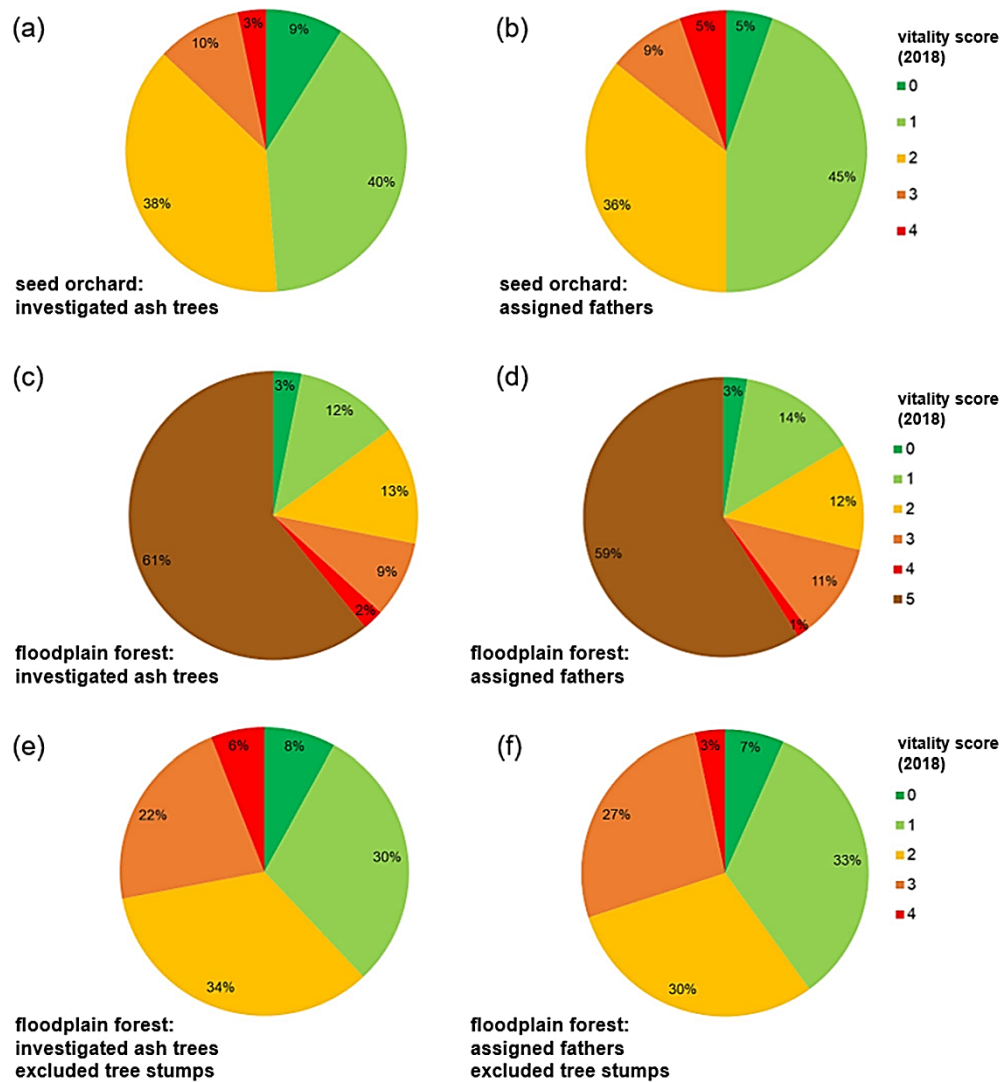


Fig. 6 Vitality of the ash trees in the study sites in 2018. Vitality of **(a)** all investigated ash trees, which could be a pollen donor versus **(b)** all assigned potential fathers in the seed orchard and **(c)** all investigated ash trees, which could be a pollen donor versus **(d)** all assigned fathers in the floodplain forest (vitality score: 0 — dark green; 1 — light green; 2 — yellow; 3 — orange; 4 — red; tree stumps with unknown vitality — brown)

healthy fathers have high pollination success. However, the effect observed by Gassner et al. (2019) that the highest ash pollen emissions occurred 1 to 2 years after the first symptoms of ash dieback noticed in a

region was very weakly perceived in our study. Similar effects of excessive flowering with increasing tree damage were also noted in the context of the forest dieback in the 1980s (Gassner et al. 2019). Thus, it

appears that *Hymenoscyphus fraxineus* infection could at least temporarily increase pollen emission. This can be explained by the fact that stress symptoms in affected trees could lead to increased flowering (Wada and Takeno 2010), which can be regarded as a compromise between reproduction and defensive reaction of the tree (Denancé et al. 2013). Since disease resistance can be partially inherited from parents to offspring (Kjær et al. 2012; Lobo et al. 2015), increased flowering of damaged trees may have a negative effect on the natural regeneration. Thus, Kjær et al. (2012) suggested that only about 1% of the ash trees have the potential to produce offspring with an expected crown damage of less than 10% under the current disease pressure. Since our study revealed low pollination success for the severely damaged father trees and we observed a poor flower development, specifically on severely damaged ash trees in previous studies (Eisen et al. 2022a), it can be assumed that severe damage is associated with decreased pollen production. Nevertheless, even severely damaged male ash trees were capable of producing offspring, which is in line with the findings of Semizer-Cuming et al. (2019; 2021). On the other hand, crown damage caused by ash dieback reduces individual reproductive success (Semizer-Cuming et al. 2021) and thus has a positive effect on natural selection. Therefore, next generations will probably develop a higher resistance to ash dieback. This emphasizes the importance of not removing healthy ash trees during thinning as well as of enriching the gene pool of existing forests through targeted planting of less susceptible ash trees.

Additionally, self-pollination does not seem to have a major effect on reproduction in ash. According to our results, only in a very small proportion of the offspring, self-pollination could be detected with high probability (one offspring in the floodplain forest). Moreover, the study by Saumitou-Laprade et al. (2018) showed that *Fraxinus excelsior* L. might be self-incompatible, preventing loss of genetic diversity, which is critical for the future of ash, especially in the light of ash dieback (Fussi 2020).

Concerning the mother trees, in the seed orchard, the majority of the offspring (52.6%) whose fathers could be identified were assigned to mothers of vitality class 1, and in the floodplain forest, the proportion was 56.3%. Since the total number of seeds produced per tree was not investigated at this point, it is not possible to make a statement about the relationship between health status and reproductive success of the mother trees. However, a couple of studies previously reported that female ash trees severely affected by ash dieback produced much lower amounts of seeds compared to healthy ones (Semizer-Cuming et al. 2019, 2021). Overall, our research indicates

a relationship between reproductive success and health, with healthy and only slightly damaged male ash trees (vitality score ≤ 2) contributing more to pollination.

4.2 Estimation of pollen transport distances

Our results showed that ash trees in Schorndorf had the highest pollination success rate (70%) within a radius of 100 m. In the floodplain forest, over 50% of pollination success occurred within 140 m, whereas only about 8% of father trees had pollination success over distances of more than 400 m. The longest detected distance of pollination in the floodplain forest was more than 550 m, corresponding nearly to the maximum extent of the study area. Thus, our findings are very consistent with previous research; the study by Heuertz et al. (2003), which revealed that effective pollen transport occurred at distances ranging from 70 to 140 m, fits well with our results from Schorndorf. The highest amount of successful pollination occurred at a distance of 76 m between father and mother trees in the seed orchard. In contrast, Semizer-Cuming et al. (2021) reported that 50% of effective pollen transport occurs within 140 m. This observation was reflected in the results of efficient pollen transport in the floodplain forest. In both cases, a sub-area of a large contiguous mixed forest was investigated, where ash occurs with other forest tree species.

Despite the fact that 2018 was a masting year at the Schorndorf seed orchard (Eisen et al. 2022a), a 66.5% foreign pollen input was detected. Seed orchards are collections of breeding populations that have been carefully selected to produce high-quality reproductive material (Fussi et al. 2014). Therefore, pollen input from ash trees from outside the orchard can affect seed quality. A seed orchard should be distinguished above all by the fact that the selected plus trees within the orchard mutually fertilize each other, with preferably no foreign input. Although no ash trees were found in the immediate vicinity of the orchard, it is possible that ash pollen from the further surrounding area contributed to fertilization. This has already been demonstrated in previous studies on effective pollen transport, which detected distances between 1.3 and 2.9 km (Bacles and Ennos 2008; Semizer-Cuming et al. 2021). In the 300-ha landscape heavily deforested described by Bacles and Ennos (2008), pollen immigration represented also between 43 and 68% of effective pollination (depending on the assignment method). Furthermore, Semizer-Cuming et al. (2017) found that 26–45% of pollen were transported from outside when they studied the genetic connectivity of ash trees in an isolated forest site in a fragmented landscape (2 ha). The cause of not local or even long-distance pollen transport could be wind related, which, as already demonstrated in other studies, is the primary factor

responsible for the dispersal of pollen (Laaidi 2001; Eisen et al. 2022a). According to Puc (2012), larger effective pollen transport distances are linked to stronger winds. Semizer-Cuming et al. (2017) found a positive correlation between prevailing wind direction and the mean direction of pollen dispersal in ash. In addition, factors such as tree height and canopy width also influence pollen dispersal (Adams-Groom et al. 2017). Thus, pollination within the orchard may have suffered from many dead trees due to ash dieback. Thus, spacing between trees increased, possibly leading to an increasingly mixed pollen cloud and higher pollen income from outside to pollinate the flowers. This also corresponds well with our pollen dispersal patterns: In the case of mother trees in marginal positions, a greater pollen input from outside was observed, especially in the orchard. Therefore, seeds should be harvested preferably from central trees having a higher probability that both parents originate from plus trees within the orchards. In Germany, the Forest Reproduction Act (FOVG 2002; BGBl. I.S. 1658) recommends that seed orchards maintain a distance of 400 m from phenotypically weak stands of the same species or a species that can be crossed with it (gGA 2019). Thus, one measure to increase pollination within the orchard is to ensure that the distance between foreign ash trees and the seed orchard is substantially greater than 400 m. In addition, protecting seed orchards primarily with conifers such as spruce planted at the borders may aid in reducing pollen input from the outside and avoiding cross-pollination. Deciduous trees or shrubs might also reduce pollen input but are more permeable because leaf formation often begins after flowering of ash or are low in height (in case of shrubs). Another fact is the synchronization of flowering time between clones in the seed orchard. Flowering of male and female flowers has to occur approximately at the same time in order to fertilize successfully. When selecting plus trees from different regions, the flowering time of the plus trees combined in the seed orchard may differ from each other. If there is only little overlap between the flowering time of male and female flowers, pollen from outside may be more fertile at certain times (Mondal et al. 2019). Therefore, we recommend that a large orchard is established with as many trees as possible. This increases the likelihood that the trees flower synchronously and thus ensures that most pollination will occur within the seed orchard.

In the floodplain forest, only 26.1% of the seeds could be assigned to their fathers, implying that pollen are transported over distances greater than 550 m. However, in the approaches based on paternity analyses, it is often difficult or infeasible to sample all trees in a forest that represent a potential pollen source for

reproduction (Baçles and Ennos 2008). Hence, it must be noted that a higher number of parent trees would possibly also shift the mean values, maxima and minima. However, as already discussed, since healthy and only slightly damaged male ash trees (vitality score ≤ 2) contribute more to pollination, there is a chance for gene flow between somewhat isolated individuals or groups of healthy ash trees. Increasingly fragmented ash populations that are expected in the future, as well as already isolated ash trees that are less susceptible to *H. fraxineus*, may still be in genetic exchange with one another, increasing the chance of a healthier next generation. Individuals of the natural regeneration are the result of natural selection and might be able to withstand inter- or intraspecific competition and could therefore be disease resistant (Metzler et al. 2012). Thus, in the long term, those genotypes that can better withstand the negative effects of the fungus will prevail and have the highest fitness under the prevalent environmental conditions (Fussi et al. 2014). However, Buchner et al. (2022) found that the environmental conditions prevailing during long-distance pollen transport play an important role in the successful pollination of female ash flowers. It was demonstrated that pollen viability decreased faster with increased or prolonged UV radiation and under warmer conditions. Instead, viable pollen could still be observed after 28 days at moderate temperatures. Thus, prolonged heat extremes caused by climate change can have serious consequences for the pollen's viability during long-distance pollen transport. Nevertheless, it has been shown that gene flow can occur over longer distances, and successful pollination is possible.

5 Conclusion

In summary, it can be concluded that effective pollen transport occurs over long distances, although with decreasing pollination success. There is still hope for the future of ash since healthy and slightly diseased fathers make a greater contribution to pollination, meaning that gene flow will facilitate the healthier next generation of ash in the future. However, in order to reduce pollen input from outside and avoid cross-pollination on the seed plantations, it is important to establish large seed orchards protected by planting around the border. In addition, female trees should be planted mainly in the center of the orchard to enhance short-distance pollination within the orchard. We recommend harvesting seeds only during masting years due to intensified pollination between plus trees in the orchard.

Appendix

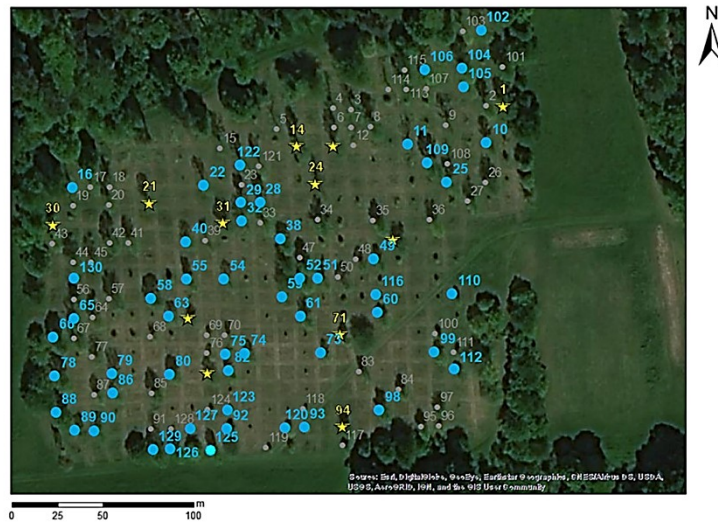


Fig. 7 Seed orchard including investigated ash trees, labeled by tree ID: yellow stars – mother trees, blue dots—ash trees identified as potential father trees by their clone number; gray dots—investigated ash trees excluded as pollen donors. Source of the map: ESRI Data & Maps

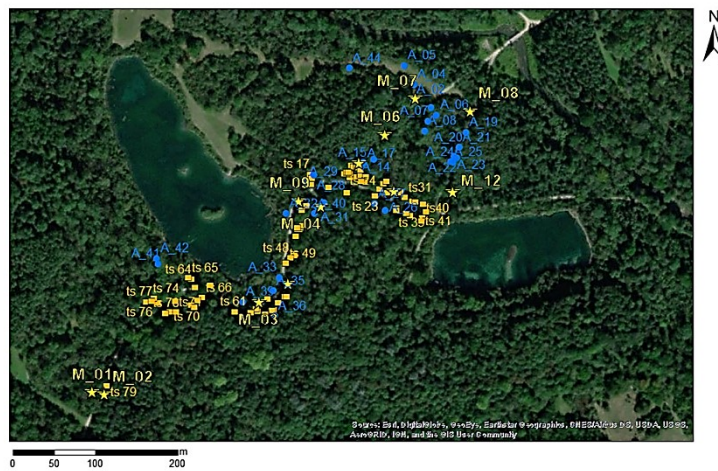


Fig. 8 Study area of floodplain forest including investigated ash trees, labeled by tree ID: yellow stars — mother trees, blue dots — ash trees identified as father trees, and orange squares — logged ash trees identified as father trees. Source of the map: ESRI Data & Maps

Table 2 Ash trees of the seed orchard near Schorndorf

Clone number	Tree-ID	New date assignment (#sisters)	Gender	Flowered	Vitality score (2012)
1877	1		f	-	3
1866	2		f	-	3
1877	3		f	-	3
1869	4		f	-	3
1877	5		h	-	3
1866	6		f	-	3
1880	8		m	-	3
1878	9		f	-	3
1880	10		m	-	3
1874	11		m	-	3
1878	12		m	-	3
1888	14		f	-	3
1878	15		f	-	3
1879	18		f	-	3
1878	19		m	-	2
1880	20		m	-	2
1877	21		f	-	3
1878	24		h	-	3
1879	25		m	-	4
1880	26		f	-	3
1888	25		m	-	1
1877	26		f	-	3
1866	27		h	-	1
1880	28		m	-	1
1880	29		m	-	1
1866	30		f	-	3
1879	31		f	-	1
1880	32		m	-	2
1874	33		f	-	2
1866	33		f	-	2
1877	34	1866	h	-	3
1874	35		f	-	1
1880	35		m	-	1
1880	36		m	-	1
1866	38		f	-	1
1866	39		f	-	1
1866	40		f	-	1
1880	41		m	-	2
1881	43		m	-	3
1880	43		m	-	2
1880	44		m	-	2
1881	45		m	-	3
1881	45		m	-	3
1877	45		f	-	1
1879	46		f	-	1
1878	49		m	-	2
1881	50		m	-	1
1880	51		m	-	3
1867	52		m	-	3
1879	54		m	-	1
1879	55		h	-	1
1877	56		h	-	1
1874	57		m	-	1
1881	58		m	-	1
1881	59		m	-	1
1880	60	1830	m	-	1
1880	61		m	-	1
1877	62		f	-	1
1845	63		f	-	1
1879	64		f	-	1
1844	65		m	-	3
1844	66		m	-	3
1863	67		f	-	1
1864	68		f	-	1
1864	69		f	-	1
1863	70		f	-	1
1864	71		f	-	0
1869	71		h	-	1
1878	74		h	-	1
1879	75		h	-	2
1877	76		f	-	2
1879	77		f	-	1
1880	76		m	-	1
1888	79		m	-	1
1880	81		m	-	1
1881	81		m	-	1
1881	81		m	-	1
1879	82		m	-	3
1878	83		f	-	1
1878	84		f	-	1
1879	85		h	-	3
1864	87		f	-	1
1881	88		m	-	2
1880	90		h	-	3
1866	91		f	-	1
1880	92		m	-	1
1868	93		h	-	1
1863	94		h	-	0
1864	95		f	-	1
1866	96		f	-	2
1879	97		f	-	2
1869	98		m	-	1
1869	99		m	-	1
1888	100		f	-	1
1881	101		f	-	1
1880	102		m	-	1
1869	103		f	-	2
1845	104	1830	m	-	1
1851	105		m	-	1
1849	106		m	-	1
1877	107		f	-	1
1879	108		f	-	2
1863	110		h	-	1
1877	111		m	-	1
1864	112		m	-	1
1879	113		f	-	1
1871	114		m	-	4
1880	115		f	-	1
1880	116		m	-	0
1879	117		f	-	1
1877	118		h	-	1
1866	(119)		m	-	0
1880	120		m	-	0
1864	121		f	-	3
1881	122		m	-	0
1880	(123)		m	-	0
1858	(124)		m	-	0
1888	125		m	-	0
1878	126		m	-	2
1870	(127)		m	-	4
1880	128		m	-	0
1881	129		m	-	1
1881	130		m	-	1

Gender assignments (m, male; f, female; h, hermaphrodite), the presence of flowers on the 109th day of the year (+ or -), and vitality classifications according to Lenz et al. (2012) (categories 0 and 1, healthy; categories 3–4, diseased; category 5, dead). Color codes are as follows: pink indicates the mother trees, blue shows the potential fathers assigned to offspring, green denotes the potential fathers unassigned to any offspring, and white shows all other ash trees excluded as potential fathers

Table 3 Ash trees of the floodplain forest

tree-ID	gender	vitality score (2012)
M_081	f	3
M_082	h	2
M_083	h	2
M_084	f	2
M_085	f	2
M_086	f	2
M_087	f	0
M_088	h	2
M_089	h	2
M_090	h	2
M_091	h	2
M_092	h	2
A_093	m	2
A_094	h	2
A_095	h	2
A_096	m	2
A_097	m	1
A_098	m	2
A_099	f	4
A_100	f	2
A_101	f	2
A_102	h	2
A_103	h	2
A_104	h	2
A_105	m	2
A_106	m	2
A_107	m	2
A_108	m	2
A_109	m	2
A_110	m	2
A_111	m	2
A_112	m	2
A_113	m	2
A_114	m	2
A_115	m	2
A_116	m	2
A_117	m	2
A_118	m	2
A_119	m	2
A_120	m	2
A_121	m	2
A_122	m	2
A_123	m	2
A_124	m	2
A_125	m	2
A_126	m	2
A_127	m	2
A_128	m	2
A_129	m	2
A_130	m	2
A_131	m	2
A_132	m	2
A_133	m	2
A_134	m	2
A_135	m	2
A_136	m	2
A_137	m	2
A_138	m	2
A_139	m	2
A_140	m	2
A_141	m	2
A_142	h	2
A_143	h	2
A_144	h	2
A_145	h	2
A_146	h	2
A_147	h	2
A_148	h	2
A_149	h	2
A_150	h	2
A_151	h	2
A_152	h	2
A_153	h	2
A_154	h	2
A_155	h	2
A_156	h	2
A_157	h	2
A_158	h	2
A_159	h	2
A_160	h	2
A_161	h	2
A_162	h	2
A_163	h	2
A_164	h	2
A_165	h	2
A_166	h	2
A_167	h	2
A_168	h	2
A_169	h	2
A_170	h	2
A_171	h	2
A_172	h	2
A_173	h	2
A_174	h	2
A_175	h	2
A_176	h	2
A_177	h	2
A_178	h	2
A_179	h	2
A_180	h	2
A_181	h	2
A_182	h	2
A_183	h	2
A_184	h	2
A_185	h	2
A_186	h	2
A_187	h	2
A_188	h	2
A_189	h	2
A_190	h	2
A_191	h	2
A_192	h	2
A_193	h	2
A_194	h	2
A_195	h	2
A_196	h	2
A_197	h	2
A_198	h	2
A_199	h	2
A_200	h	2

Gender assignments (m, male; f, female; h, hermaphrodite), the presence of flowers on the 109th day of the year (+ or -), and vitality classifications according to Lenz et al. (2012) (categories 0 and 1, healthy; categories 3–4, diseased; category 5, dead). Color codes are as follows: pink indicates the mother trees, blue shows the potential fathers assigned to offspring, green denotes the potential fathers unassigned to any offspring, and white shows all other ash trees excluded as potential fathers

Table 4 Specifications of the fifteen nuclear microsatellite markers used in the study. Markers with no reproducible results are shown in Italics

Primer name	Temp. [°C]	Length [bp]	Label	Multiplex	Repeat motif	Literature
M2-30	55	182–294	Dy751	A	(TG)15(AG)23	Brachet et al. (1999)
<i>Fp12353</i>	55	218–243	Cy5	A	(TAAT)5	Noakes et al. (2014)
<i>Fp21068</i>	55	232–282	Cy5	A	(TTGGT)4	Noakes et al. (2014)
<i>Fp19681</i>	55	209–222	Dy751	A	(CT)8	Noakes et al. (2014)
Fp18437	55	302–321	Dy751	B	(CT)10	Noakes et al. (2014)
Femsatl 11	55	161–234	Cy5	B	(GA)20(TA)4	Lefort et al. (1999)
Femsatl 12bis	55	147–261	Dy751	B	(GA)6CA(GA)8	Gerard et al. (2006)
Femsatl 4	55	155–205	Cy5	B	(CA)2(AG)24	Lefort et al. (1999)
Fp14665	55	260–297	IRD700	B	(CT)8	Noakes et al. (2014)
Fp21064	59	234–241	Cy5	C	(GCC)7	Noakes et al. (2014)
FRESTSSR308	59	194–202	Dy751	C	(AG)8	Aggarwal et al. (2011)
<i>FRESTSSR427</i>	59	224–272	IRD700	C	(TAAT)4	Aggarwal et al. (2011)
FRESTSSR528	59	271–304	Cy5	C	(TTC)4/(CTG)8	Aggarwal et al. (2011)
Femsatl 19	59	142–238	IRD700	C	(CA)6CGGC(CA)13	Lefort et al. (1999)
ASH2429	59	235–255	Dy751	C	(TTC)7	Bai et al. (2011)

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Authors' contributions

Conceptualization, BF and SJO; methodology, BF, DSC, SJO, and AKE; formal analysis and investigation, AKE; data curation, AKE; visualization, AKE; writing — original draft preparation, AKE; writing — review and editing, AKE, SJO, BF, and DSC; funding acquisition, SJO and BF; and supervision, SJO. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations**Ethics approval and consent to participate**

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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6.3 Does ash dieback affect the reproductive ecology of *Fraxinus excelsior* L.?

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ORIGINAL PAPER

Does ash dieback affect the reproductive ecology of *Fraxinus excelsior* L.?

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Abstract Forest tree species reproduction is a key factor in maintaining the genetic diversity of future generations and the stability of forest ecosystems. The ongoing ash dieback disease could affect the reproductive ecology of *Fraxinus excelsior* L. and have a major impact on the quantity and quality of pollen and seeds. In this study, we investigated pollen production and viability of pollen and seeds of ash trees with different health status from 2018 to 2022. Inflorescences were collected from 105 trees (pollen production), pollen from 125 trees (pollen viability), and seeds from 53 trees (seed quality) in two seed orchards and in one floodplain forest in southern Germany. Not all parameters were examined at every site every year. The average pollen production per tree was estimated at 471.2 ± 647.9 billion pollen grains. In addition, we found that a high number of inflorescences did not equate to high pollen production per inflorescence. Pollen production of healthy and diseased trees did not differ significantly, although only 47% of severely diseased male trees (vs. 72% for healthy trees) produced flowers. With regards to pollen viability, the TTC test showed

an average viability of $73\% \pm 17\%$. Overall, there was a slight tendency for diseased trees to have less viable pollen. However, a significant difference could only be calculated for trees in the floodplain forest. The percentage of germinable seeds in 2018 was 38% in the floodplain forest and 57% in one of the seed orchards. The percentage of viable seeds (TTC test) ranged from 17 to 22% in the orchards in 2020. Non-viable seeds were usually heavily infested by insects. In general, seed quality was not significantly different between healthy and diseased trees. Our results indicate that ash dieback affects flower formation and pollen viability but not pollen production or seed quality. Nevertheless, the fact that hardly any flowering was observed, especially for trees that were seriously affected, suggests a negative effect of ash dieback on reproductive performance. Thus, severely diseased trees will transfer their genes to a smaller extent to the next generation.

Keywords Common ash · Seed orchard · Floodplain forest · Pollen production · Viability · TTC test · Seed stratification · Phenology

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Introduction

The reproduction of forest tree species is critical to the health and sustainability of forest ecosystems (Hammond et al. 2021). It contributes to species conservation but also to natural selection and genetic diversity under prevailing environmental conditions (Smith 1981; Jump et al. 2009; Isabel et al. 2020; Wang et al. 2022). The potential for adaptation is particularly important with regards to the long generation time in natural forest landscapes, where environmental conditions may change due to factors such as climate change, diseases and/or pest infestations (Pautasso et al.

2010; McKinney et al. 2014; Isabel et al. 2020). Since sexual reproduction in trees generally occurs through pollination of female flowers by male pollen, pollen production is critical in passing on genetic information to the next generation (Smith 1981; LaDeau and Clark 2006; Fussi et al. 2014; Larue et al. 2021). The majority of forest tree species are anemophilous plants (LaDeau and Clark 2006). In contrast to insect-pollinated species, which produce distinct flowers, trees that rely on wind pollination produce a large number of flowers and pollen (Holsinger and Steinbachs 1997; Dellinger 2020; Timerman and Barrett 2020). This increases the chances of a pollen grain encountering a female flower. In years when a particularly large number of flowers and pollen is produced, the probability of successful pollination increases considerably. This has an effect on seed production patterns and explains why annual fluctuations in pollen production affect masting years of seed production (Satake and Iwasa 2002; LaDeau and Clark 2006; Mangla and Gupta 2015). In addition to quantity, quality is essential for successful pollination (Buchner et al. 2022), and results in the formation of a new genotype in a new seed. The genotypes that spread in a healthy ecosystem are primarily those with the highest fitness due to evolutionary adaptation (Fussi et al. 2014). However, a variety of environmental conditions and diseases can have an impact on tree reproductive processes. Duro et al. (2013) discovered that air pollutants such as carbon monoxide (CO), sulfur dioxide (SO₂), and nitrogen dioxide (NO₂) reduce pollen viability. Increased ozone (O₃) concentrations also have a negative effect on tree reproduction and regeneration (Darbah et al. 2008). In contrast, rising carbon dioxide (CO₂) levels in the atmosphere promote flower and pollen reproduction as well as seed germination and viability in some tree species (LaDeau and Clark 2006; Darbah et al. 2008). Climate change will exacerbate air pollution levels through indirect impacts, increased energy consumption, ground-level ozone generation, forest fires (Luschkova et al. 2022; IPCC 2023). Other factors such as extreme weather conditions (heat, cold, drought, heavy precipitation), which are expected to become more frequent and/or intense, will also affect plant reproductive biology and influence, e.g., the stamens (Ohnishi et al. 2010; Hedhly 2011; Carpenedo et al. 2017; Talwar et al. 2022). Further, investigations on the relationships between temperature and pollen production reveal that high air temperatures increased pollen production (D'Amato et al. 2007; Zhang and Steiner 2022). In addition, diseases and pathogens are causing current damage to forest trees, ranging from biodiversity loss to changes in biochemical cycling (Aber et al. 2001; Lindner et al. 2014; Liebhold et al. 2017; Balla et al. 2021; Cotrozzi 2022). As shown by Kozłowski (1971), this can affect the vigour of forest trees and their ability to flower and produce fruits.

Ongoing ash dieback could also have an impact on the reproductive ecology of *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl (Gross et al. 2014). The disease is caused by the fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya (Baral et al. 2014) and its asexual stage *Chalara fraxinea* (Kowalski 2006), which acutely threatens the population of common and narrow-leaved ash (Metzler et al. 2012; Gross et al. 2014; McKinney et al. 2014; Enderle 2019). According to simulations, the increasing damage to shoots and wood, as well as the high mortality rates caused by the fungus (Pautasso et al. 2013; Plitřra et al. 2017; Tulik et al. 2018; Semizer-Cuming et al. 2021), will lead to a decline of up to 75% of the population in the next 30 years (Coker et al. 2019). However, since natural variability in ash susceptibility has been observed, it is important to establish a more resilient next generation to ensure their future (Semizer-Cuming et al. 2017, 2019; Fussi 2020). The current literature indicates that resistance to ash dieback is inherited rather than linked to population or source (McKinney et al. 2011, 2014; Enderle et al. 2017; Semizer-Cuming et al. 2019). Thus, disease development at the stand level is dependent on less susceptible genotype ability to spread their genes via pollen and seed (Lobo et al. 2015; Semizer-Cuming et al. 2019, 2021). Due to the large-scale decline of the populations, it is also necessary that the ash trees are sufficiently linked with each other and produce viable pollen, which can be transported over long distances (Buchner et al. 2022; Eisen et al. 2022).

Common ash is a wind-pollinated, polygamous deciduous species whose inflorescences may be female, male, or both, and whose gender expression can vary from year to year (Douglas et al. 2013; Bajc et al. 2020). Sexual maturity occurs between the ages of 15 and 30 years in open stands and between 30 and 35 years in closed stands (Roloff and Pietzarka 1997; Douglas et al. 2013). Due to its hermaphroditism, it is able to self-fertilize but these seeds are unlikely to survive due to inbreeding depression (Saumitou-Laprade et al. 2018; Bajc et al. 2020). Mature seeds drop in late autumn and remain dormant for two to six winters before germinating (Douglas et al. 2013; Bajc et al. 2020). Since pollen and seeds are both dispersed by wind, fragmented ash populations result in limited gene flow and pollination success (Fussi et al. 2014; Eisen et al. 2022). Heuertz et al. (2003) determined a mean seed dispersal distance of 14 m in a mixed deciduous forest in Romania, and Morand et al. (2002) suggested 140 m in continuous forests across France. Similar results were obtained from studies on pollen transport. In a study of aerobiological pollen transport in seed orchards in southern Germany, 50% of pollen dispersal was within 200 m (Eisen et al. 2022). In another study on effective pollen transport, average pollination success ranged from 76 to 166 m (Eisen et al. 2023).

Castiñeiras et al. (2019) investigated the production and viability of *Fraxinus* pollen in Spain, regardless of ash dieback. They estimated average pollen production per anther of *F. excelsior* L. to be 27,664 pollen grains. The average viability of pollen was 31.9% (derived from TTC staining). Furthermore, they detected a significant increase in annual pollen over the last few decades. According to Gassner et al. (2019), the highest pollen emissions occurred one to two years after ash dieback was observed and this could not be explained by the trees natural rhythm. Thus, ash dieback appears to increase pollen production and hence pollen concentrations in the air; however, whether this is a short-term effect has not been investigated. Similar effects of excessive blooming under increased tree damage have been discovered in the forest dieback of the 1980s (Gassner et al. 2019). Consequently, stress conditions can trigger an “emergency response” and lead to a stronger inflorescence production (Wada and Takeno 2010). Effects of ash dieback on pollen quantity and quality have direct effects on reproduction and thus on seeds, as reduced pollen quality will reduce the ability of pollen to fertilize flowers.

Investigations have yet to determine the extent the pathogen influences the quality of male gametes. It is possible that the production and viability of pollen is altered in diseased trees but this has not been determined. Studies on seed quality are also particularly important, as the seeds should produce offspring with high resistance to ash dieback, sufficient genetic diversity, and good growth characteristics (Bubner et al. 2017). Studies show that seed viability of ash is about 50% after 15 to 20 years; thus, seed storage is considered beneficial (Fussi et al. 2017). In addition, seeds from less susceptible mother trees may be suitable for genetic conservation or breeding programs (McKinney et al. 2014). Thus, the cultivation of these seedlings could be a great opportunity for endangered ash.

It was hypothesized that pollen and seed quality of common ash decreases with increasing infection, while pollen production may increase in the short term as a stress response. The objective of this study was to investigate whether ash health status has an impact on pollen and seed quality as well as pollen production at different sites under different environmental and site conditions.

Materials and methods

Study areas and vitality assessment

Ash inflorescences, pollen and seeds were collected from two ash seed orchards and one natural floodplain forest in southern Germany (Fig. 1 a). The seed orchard trees are clone graftings of plus trees selected with respect to growth

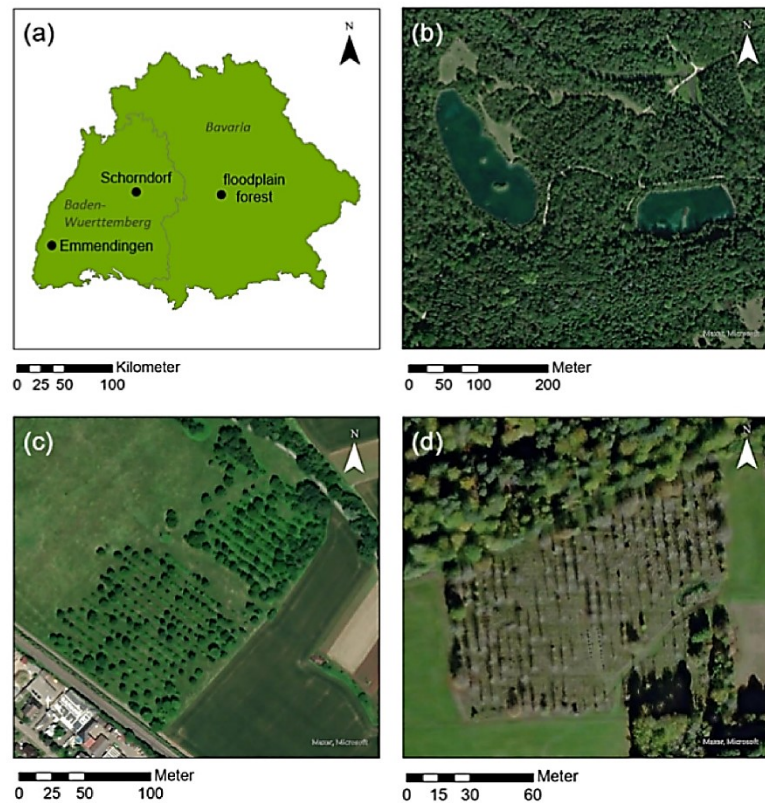
and stem quality before the outbreak of ash dieback (FVA 2006; Enderle et al. 2015). The floodplain forest is a natural landscape in which common ash has a share of approximately 15% (Jochner-Oette et al. 2021; Eisen et al. 2023).

The floodplain forest of approximately 10 ha is located at the Danube near the Bergheim barrage near Ingolstadt, Bavaria (48°44' N, 11°16' E, 375 m a.s.l.). (Fig. 1 b). Average annual temperature is 7.8 °C and average annual precipitation 715 mm (1961–1990) (Schwab et al. 2018). Heights of the trees varied between 10 and 30 m (average 22 m). Average tree circumference was 123 cm.

The Emmendingen seed orchard of 4.3 ha (48°7' N, 7°52' E, 210 m a.s.l.) is located in the north-west of the industrial area of Emmendingen in Breisgau, Baden-Württemberg (Fig. 1 c). Average annual temperature is 10.2 °C and average annual precipitation 882 mm (DWD station “Emmendingen-Mundingen”, 1981–2010). The seed orchard was established in 1995 with 49 clones (3–6 ramets per clone, 228 trees in total) (Eisen et al. 2022). The grafted plus trees originated from the southern German hill and mountain areas (Enderle et al. 2015). Due to the wide plot size (7.5 m × 10 m to 15 m × 15 m), no thinning measures have been carried out. Nevertheless, 142 trees were removed due to ash dieback, and isolated crown pruning was carried out in the course of harvesting measures (FVA 2006). In July 2020, 86 mature trees remained in the orchard, of which 33 (38%) were classified as healthy, 52 (60%) as damaged and one as dead. In July 2021, 84 were still alive, with only 32 (37%) classified as healthy. Heights ranged from 6 to 18 m, with most between 11 and 14 m tall. Average tree circumference was 88 cm.

The Schorndorf seed orchard (48°46' N, 9°25' E, 420 m a.s.l.) is located in the Rems valley about 20 km east of the city of Stuttgart, Baden-Württemberg, and covers an area of approximately 2.3 ha (Fig. 1 d). Average annual temperature is 10.3 °C (DWD station “Stuttgart-Schnarrenberg”, 1981–2010) and average annual precipitation 855 mm (DWD station “Winterbach, Rems-Murr-Kr.”, 1981–2010). It was established in 1992 with 68 clones (4–8 ramets per clone, a total of 416 trees) in a 7 m × 7 m plot design. The clones originated from the hilly and mountainous areas of southern Germany as well as from the Alps and the Alpine foothills (Enderle et al. 2015). The majority of trees (approx. 70%) died due to ash dieback (Eisen et al. 2022, 2023). By the beginning of our surveys in 2018, 123 mature trees remained in the seed orchard and 58 (47%) were classified as healthy. In July 2020, 34 (28%) were classified as healthy, 79 (65%) as damaged and 7% were dead. The number of healthy trees stabilized at 28% in 2021, 62% were classified as damaged and 10% as dead. The height of the ash trees varied between 3 and 17 m (average 11 m). The average tree circumference was 64 cm.

Fig. 1 **a** location of the three study sites in Bavaria and Baden-Wuerttemberg, southern Germany; **b** area of the floodplain forest; **c** seed orchard near Emmendingen; **d** seed orchard near Schorndorf. Source of maps: ESRI Data & Maps



All trees were classified annually (2019–2021) in July according to a 6-grade scoring key assessing the vitality of mature trees with regards to ash dieback (Lenz et al. 2012). Characteristics for quantifying the degree of damage are leaf loss, dead shoots, and the formation of a secondary crown (Table 1). Trees in categories 0 and 1 (up to max. 30% leaf loss and only slight disease symptoms) were classified as healthy, all other categories were diseased (2–4) or dead (5). In addition, weekly phenological observations were carried out in spring according to the

BBCH code (Meier 2001) to determine the optimal time for collecting inflorescences and pollen.

Pollen production

Shoots with male inflorescences were collected from mid-March to early April in the seed orchards in 2020 and 2021. It was determined that the pollen were mature but the anthers closed. The trees were selected based on the accessibility of the shoots and the presence of male inflorescences. The number of trees examined (105) and their health status per

Table 1 Vitality score to classify the health of mature ash trees in terms of ash dieback according to Lenz et al. (2012)

Vitality score	Symptom description
Vitality score 0	≤ 10% leaf loss; healthy tree crown
Vitality score 1	≤ 30% leaf loss; dead shoot tips only on the outer crown
Vitality score 2	≤ 50% leaf loss; crown with sparse foliage, dead shoots
Vitality score 3	≤ 80% leaf loss; secondary crown development, first branches dead
Vitality score 4	≤ 99% leaf loss; many dead branches, only residual foliage
Vitality score 5	100% leaf loss, dead tree

Table 2 Number of ash trees examined for pollen production and their health status per study site and year; trees in categories 0 and 1 are healthy, all other categories are diseased (2–4)

Study site	Number examined			
	Schorn- dorf 2020	Schorn- dorf 2021	Emmending- gen 2020	Emmending- ingen 2021
Vitality score 0	–	1	–	–
Vitality score 1	8	5	11	17
Vitality score 2	8	13	12	14
Vitality score 3	1	4	1	5
Vitality score 4	–	5	–	–
Total	17	28	24	36

study area and year are shown in Table 2. The samples were taken from different shoots per tree at 1.5 m to 5 m above the ground from all cardinal directions. After sampling, the material was preserved at $-20\text{ }^{\circ}\text{C}$ until further processing.

To calculate pollen production at larger scale levels, e.g., per individual tree, the number of inflorescences per m^3 of the crown ($50\text{ cm} \times 50\text{ cm} \times 50\text{ cm}$; 0.125 m^3) was counted as a random sample (Damialis et al. 2011). In addition, the circumference of the trunk was determined at breast height with a tape measure and the height and crown with a laser rangefinder (Forestry Pro II, Nikon, Chiyoda, Tokyo, Japan). Crown diameter was calculated by averaging two perpendicular diameters at its widest point. For total crown volume, an oval crown was assumed (Nelson 1997).

The laboratory analysis was based on the methods of Damialis et al. (2011) but slightly modified for the analysis of ash pollen (Ranpal et al. 2022). For each year, one inflorescence of average size was selected per tree from each cardinal direction. Each were separated from the shoot and the anthers counted. Anthers per inflorescence were soaked in 1% potassium hydroxide (KOH) solution (Moore 1999; Fægri 2000). After 24 h, the samples were heated on a hotplate at $150\text{ }^{\circ}\text{C}$ until the KOH solution had almost evaporated. The remaining tissue was broken with a mortar to extract the pollen from the anthers. To color the pollen and avoid clumping (Shivanna and Rangaswamy 1992), a 2 mL safranin solution and 70% glycerol solution was added to a volume of 20 mL. Using a VITLAB® micropipette, two samples of 10 μL per suspension (25 mL) were taken under vigorous stirring, to ensure homogeneity of the solution, and placed on each slide. After covering with a cover glass, the pollen grains were counted under a light microscope (Axio Lab A1, Carl Zeiss AG, Oberkochen, Germany) at $100\times$. In case of clumping of pollen on the slides, the procedure was repeated.

Pollen production was calculated at four levels (1) inflorescence (P_{inf}); (2) anthers level (P_{fl}), (3) volume per m^3 of crown (P_{vol}) and (4) individual tree (P_{in}) based on Damialis

et al. (2011) and Ranpal et al. (2022) using the following equations:

$$P_{\text{inf}} = \frac{V_{\text{su}}}{V_{\text{sa}}} \bar{p} \quad (1)$$

$$P_{\text{fl}} = \frac{P_{\text{inf}}}{\text{fl}} \quad (2)$$

$$P_{\text{vol}} = P_{\text{inf}} \frac{C_{\text{su}}}{M} \quad (3)$$

$$P_{\text{in}} = P_{\text{vol}} \times V \quad (4)$$

where V_{su} is the volume of the suspension (25 mL), V_{sa} the volume of the sample, \bar{p} the average number of pollen grains per 10 μL of suspension, fl the average number of anthers per inflorescence per tree, C_{su} the average number of inflorescences per m^3 volume unit of the crown, M is the volume of the sampling unit (0.125 m^3) and V the total crown volume.

Pollen quality

The tetrazolium (TTC; 2,3,5-triphenyltetrazolium chloride) test was used to determine the viability of ash pollen (Stanley and Linskens 1974; Shivanna and Rangaswamy 1992), which was particularly suitable for *Fraxinus* pollen in studies by Castiñeiras et al. (2019) and Buchner et al. (2022). The TTC test is an enzymatic test that examines the respiratory activity of tissues and thus the activity of pollen (Iannotti 2000; Duro et al. 2013). After the redox reaction, the live cells turn red due to electrons from the respiratory chain reaction, while the red change to 1,3,5-triphenylformazan (formazan) is absent in non-developing pollen. Thus, living cells can be distinguished from the unstained dead cells (Shivanna and Rangaswamy 1992; Krämer et al. 2007).

Ash pollen were harvested from trees with male flowers at varying degrees of damage in all study areas in spring when the 125 trees were in full bloom. The trees were selected based on the accessibility of the branches and the presence of male inflorescences. As 2018 was a flower-rich year, few flowers were present in 2019. The number of trees examined and their health per study area and year are shown in Table 3.

Pollen from several open flowers of each individual were carefully spread onto a slide in a thin layer. Two drops of a 1% TTC solution consisting of 1 g 2,3,5-triphenyltetrazolium chloride and 60 g sucrose in 100 ml of distilled water were placed on the slide and covered with a cover slip. The slide was kept in the dark in a Petri dish with moist filter paper (Castiñeiras et al. 2019). For each tree, three to five replicates were made and after 24 h, the colored pollen grains were counted. Light red to dark red pollen were

Table 3 Number of trees examined for pollen quality and health status per site and year. Ash trees in categories 0 and 1 are healthy, all other categories are diseased (2–4)

Study site	Number of ash trees examined				
	Floodplain forest 2019	Schorndorf 2020	Schorndorf 2021	Emmendingen 2020	Emmendingen 2021
Vitality score 0	–	1	1	–	–
Vitality score 1	8	11	5	13	15
Vitality score 2	4	9	13	16	13
Vitality score 3	1	2	3	–	5
Vitality score 4	1	–	4	–	–
Total	14	23	26	29	33

Table 4 Number of trees examined for seed quality and their health status per study site and year; trees in categories 0 and 1 are healthy; other categories are diseased (2–4)

Study site	Number of ash trees examined			
	Floodplain forest 2018	Schorndorf 2018	Schorndorf 2020	Emmendingen 2020
Vitality score 0	1	1	–	1
Vitality score 1	4	9	6	3
Vitality score 2	5	8	5	3
Vitality score 3	2	–	2	1
Vitality score 4	–	–	2	–
Total	12	18	15	8

classified as *viable* and colorless or yellowish colored grains as *non-viable*. Pollen grains that were weakly colored and therefore not identifiable were classified as *semi-viable* (Buchner et al. 2022). Counting of pollen from these categories was carried out up to 400 grains in a random middle row of the slide using a light microscope (Axio Lab A1, Carl Zeiss AG, Oberkochen, Germany) at 40× magnification and percentages were calculated.

Seed quality

Seed collection was carried out in October 2018 (floodplain forest and Schorndorf) and in September 2020 (Schorndorf and Emmendingen) with a forklift and/or a fruit picker when the fruit and seed shells were brown, i.e., desiccation had begun. Fifty-three trees were investigated. Seeds from 2018 were originally collected for another study in which genetic analyses were used to investigate pollination success of *F. excelsior* (Eisen et al. 2023). Unfortunately, no seeds were

collected from mother trees with vitality scores 3 and 4. Table 4 shows the number of trees examined and their health status per study area and year. The seeds were analyzed for quantity and quality (full or hollow grains, insect damage and germination potential). For each tree, 400 mature seeds were examined (a total of 18,078 seeds).

Seeds from 2018 were stratified into two phases according to the *International Rules for Seed Testing* (ISTA 2003a), as ash seeds exhibit deep morpho-physiological dormancy, i.e., germination inhibition by abscisic acid (Krauss and Köhler 1985). To stimulate the underdeveloped embryos, the seeds were stored for 10 weeks at 20 °C (warm stratification) and then for seven months at 3 °C (cold stratification) in moist quartz sand in a climate chamber. Afterwards, the seeds were sieved, washed and placed in several germination trays per tree on moist cell paper in the climate chamber. For germination, the climate chamber was set at 20 °C for 16 h and 30 °C for 8 h, with UV irradiation during the 8 h to simulate a day-night cycle; humidity was 70%. After 14 days, the seeds were counted for the first time according to the following categories: germinated, not germinated, healthy and not germinated, diseased, hollow or insect damage. Seeds that were healthy and started to germinate within 56 days or whose embryos showed a length increase of 100% (Krauss and Köhler 1985) were classified as *viable*, all others were *non-viable*.

The seeds from 2020 were tested with the TTC test (see section on pollen quality) to accelerate the process despite the inhibition of germination. This method allows a faster but equally accurate determination of seed viability (Krämer et al. 2007). To assess the condition of the seeds, a species-specific evaluation scheme, according to the *ISTA Working Sheets on Tetrazolium Testing* (ISTA 2003b), which dictates the minimum extent and location of stained and thus viable tissue, was followed: The pericarp of the dry seeds was removed and the endosperm soaked in water for 18 h at room temperature. The swollen endosperm was then incised longitudinally on both sides to open the embryonic cavity. The pretreated seeds were placed in a 1% TTC solution at 30 °C for staining, which served as an indicator to show the reduction processes taking place in living cells. After another 18 h, the TTC solution was rinsed off and the endosperm split longitudinally in half using a razor blade to expose the embryo. The seed was considered viable if it was devoid of flaccid and necrotic tissue and had a red coloration in areas necessary for germ development. In addition, the categories rotten, hollow grain or insect damage were also recorded.

Data analysis

Statistical analysis was performed using the software R (RStudio version 4.1.3). The package psych (Revelle 2022) was used for calculations and the packages ggplot2

(Wickham 2016) and ggpubr (Kassambara 2023) for generating plots. Pollen production as well as pollen and seed quality data were analyzed descriptively. The Kruskal–Wallis test (> 2 variables) followed by a *post-hoc* test (Wilcoxon signed-rank test) or the Mann–Whitney–Wilcoxon test (2 variables) determined if the differences between the study parameters (pollen production, pollen and seed quality) and the years or locations, as well as the health status of the trees, were statistically significant. Suitability for these tests was determined in advance by testing the variables for normal distribution (Shapiro–Wilk test). In addition, the Spearman correlation test examined whether ash trees with high pollen production produced less viable pollen, considering here only trees from the plantations from which both inflorescences and pollen were collected (84 trees).

Results

Pollen production

Average pollen production, regardless of year and location, was 4.6 ± 2.3 million pollen grains per P_{inf} , 10.8 ± 5.1 thousand pollen grains per P_{fl} , 2.0 ± 1.6 billion pollen grains per P_{vol} , and 471.2 ± 647.9 billion pollen grains per P_{in} . Since P_{fl} , P_{vol} and P_{in} are calculated based on P_{inf} and C_{su} , we have provided more detail about P_{inf} and C_{su} in the following. Statistical analysis for P_{inf} , P_{fl} , P_{vol} , P_{in} , fl , C_{su} are in Appendix Table S1.

Average pollen production per inflorescence (P_{inf}) for 2020 was 5.2 ± 2.0 million pollen grains in Schorndorf and 5.8 ± 2.3 million pollen grains in Emmendingen (Fig. 2 a). For 2021, the average P_{inf} in Schorndorf was 2.0 ± 1.2

million pollen grains, in Emmendingen 5.0 ± 2.2 million pollen grains. Pollen production in Schorndorf was substantially lower in 2021 (–80%). In general, the P_{inf} value fluctuated strongly over the two years at both sites but was particularly strongly in 2021 (Schorndorf: min 144.375, max: 6.035.398; Emmendingen: min: 150.625; max: 9.194.063). The difference between P_{inf} in the two years at both sites was statistically significant ($p < 0.001$). The *post-hoc* test showed a significant difference between the years in Schorndorf ($p < 0.001$) and between the sites in 2021 ($p < 0.001$).

The number of inflorescences per unit volume of crown (C_{su} ; 0.125 m^3) also varied widely and significantly ($p < 0.001$) between the years (Fig. 2 b). The *post-hoc* test showed a significant difference between the years at both sites ($p < 0.001$). The average C_{su} for 2020 was 33 ± 12 inflorescences per m^3 in Schorndorf and 35 ± 18 in Emmendingen. For 2021, an average C_{su} of 59 ± 22 inflorescences per m^3 was registered in Schorndorf and 73 ± 34 in Emmendingen. This indicates that a high number of inflorescences does not equate to high pollen production per inflorescence and *vice versa*. The average C_{su} in 2020 was 44% lower in Schorndorf and 52% lower in Emmendingen than in 2021.

Pollen production per inflorescence (P_{inf}) of healthy and diseased trees was not significantly different ($p > 0.05$) (Fig. 3). For P_{fl} , P_{vol} and P_{in} , there was no statistically significant difference (Appendix Table S1/Fig. S1–S3). However, for P_{inf} and P_{fl} , there was a tendency for the median of diseased trees to be higher than trees with vitality scores of 0 and 1 (except Schorndorf 2021; Figs. 3, S1). This is, however, no longer evident at the larger scale (P_{vol} and P_{in}) (Figs. S2, S3).

C_{su} also indicated no statistically significant differences between healthy and diseased trees that bore inflorescences

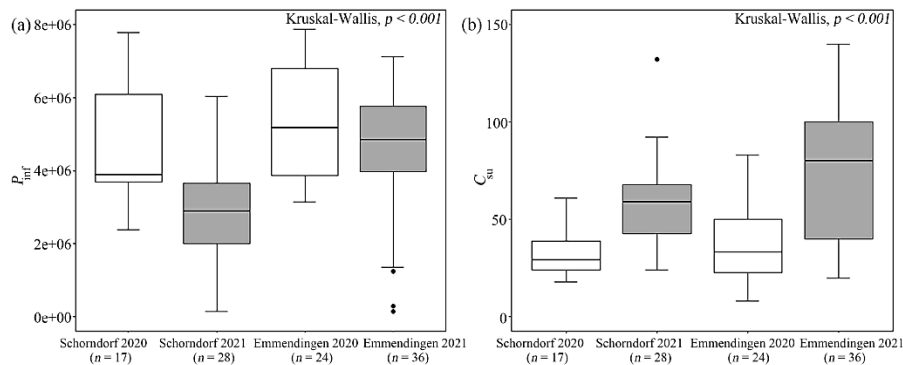


Fig. 2 Boxplots for estimated pollen production per inflorescence (P_{inf}) and number of inflorescences per 0.125 m^3 of crown (C_{su}) for Schorndorf and Emmendingen seed orchards in 2020 and 2021; n indicates number of trees sampled. The interquartile range is repre-

sented by the height of the boxes, maximum and minimum values by the upper and lower whiskers, the median by bold horizontal lines in the boxes, points indicate outliers

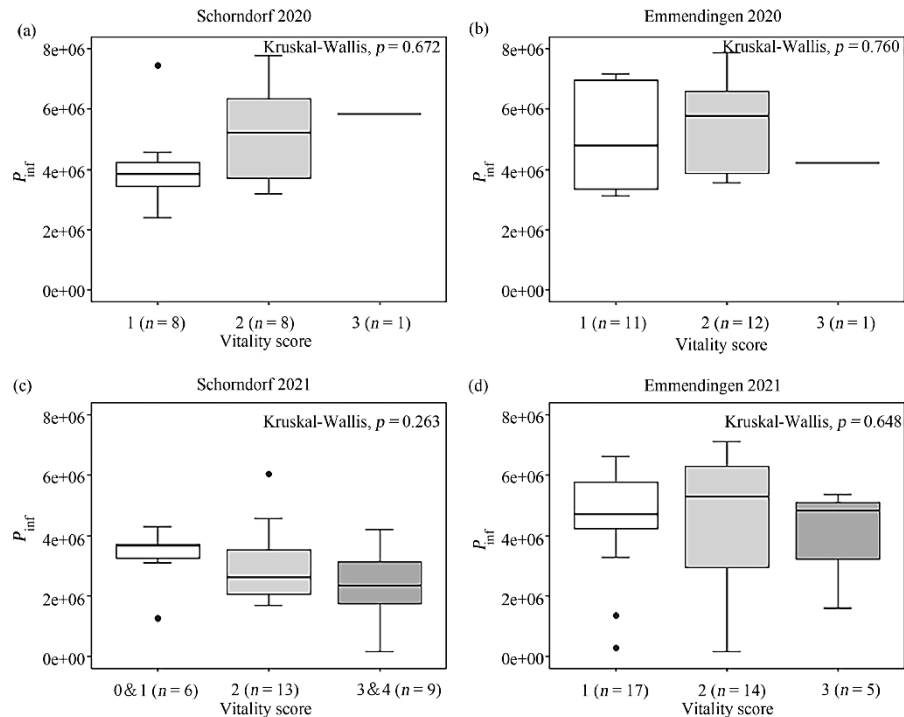


Fig. 3 Boxplots for pollen production per inflorescence (P_{inf}) classified by healthy (vitality score 0 and 1: white), diseased (vitality score 2: light grey) and severely diseased (vitality score 3 and 4: dark grey) for Schorndorf and Emmendingen seed orchards in 2020 and 2021;

n indicates the number of trees sampled; interquartile range is represented by the height of the boxes, maximum and minimum values by the upper and lower whiskers, the median by bold horizontal lines, points indicate outliers

($p > 0.05$). However, phenological observations demonstrated that only 47% of severely diseased male trees (vitality score 3: 48% and vitality score 4: 45%) developed inflorescences. For healthy trees it was 72% (vitality score 0: 33% and vitality score 1: 77%), and for mildly diseased trees, it was 61% (vitality score 2). The low percentage of healthy trees with a vitality score 0 can be explained by the fact that many were young and some perhaps not sexually mature.

Pollen quality

Pollen viability averaged $73 \pm 17\%$ for viable, $17 \pm 14\%$ for semi-viable and $10\% \pm 8\%$ for non-viable regardless of year and location. In 2019, the average viability in the floodplain forest was $79 \pm 6\%$; in 2020, $76 \pm 19\%$ in Schorndorf and $73 \pm 14\%$ in Emmendingen and in 2021, $82 \pm 12\%$ in Schorndorf and $61 \pm 21\%$ in Emmendingen (Appendix Table S2). Analysis of variance (Kruskal–Wallis test + *post-hoc* test) confirmed a statistically significant difference ($p < 0.001$) in 2021 between the study sites Emmendingen and Schorndorf

(Fig. 4). Among all sites, viability varied widely from 0.5% to 98%. The proportion of semi-viable pollen and non-viable pollen is shown in Appendix Table S2.

There was a statistically significant difference in pollen viability for different health categories for the floodplain forest in 2019 using the Kruskal–Wallis test ($p = 0.015$). However, the number of severely damaged trees (vitality scores 3 and 4) was only two. The *post-hoc* test indicated a significant difference between healthy trees in categories 0 and 1 and diseased trees in category 2 (Fig. 5 a), with diseased trees having less viable pollen. In contrast, there was no statistically significant differences for viability between the categories for 2020 and 2021 (Fig. 5 b–c). However, in 2021, the Emmendingen site showed a marginal significance with a p -value of 0.061. Also in this case, trees with a vitality score of 3 produced substantially less viable pollen than trees with vitality scores of 1 and 2 (Fig. 5 e). From a visual point of view, the mean viability differs more clearly between the different vitality scores. Thus, there is a slight tendency for

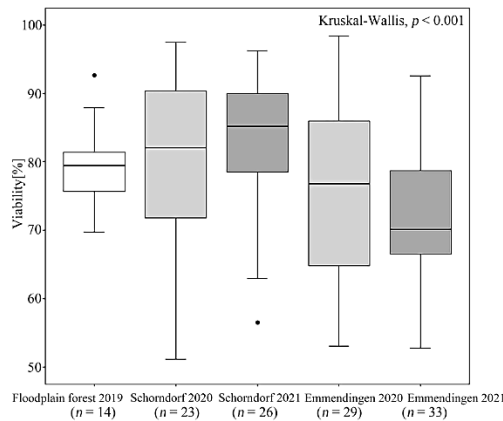


Fig. 4 Boxplots of ash pollen viability for the floodplain forest, and for Schorndorf, and Emmendingen in 2019, 2020, and 2021; *n* indicates the number of trees sampled in each site. Interquartile range is represented by the height of the boxes, maximum and minimum values by the upper and lower whiskers, the median by bold horizontal lines in the boxes, points indicate outliers

diseased trees to have less viable pollen (except Schorndorf 2020 but linked to a small sample size, *n* = 2).

The correlation between viability and pollen production per tree is shown in Fig. 6. There were no significant correlations between reproductive metrics (P_{inf} , P_{fl} , P_{vol} , P_{in}) and pollen viability using the Spearman correlation test. Accordingly, trees with high pollen production do not produce less viable pollen and vice versa.

Seed quality

In 2018, seed germination rates averaged 38% in the floodplain forest and 57% in Schorndorf. For 2020, viability values with TTC staining were lower, 22% in Schorndorf and 17% in Emmendingen (Appendix Table S3). In general, all non-viable seeds were heavily infested with insects, i.e., in 2018, 80% of non-viable seeds in the floodplain forest and 73% in Schorndorf were infested with insects. For 2020, the proportion of insect-infested seeds was 21% in Schorndorf and 15% in Emmendingen. Comparison with the Mann–Whitney U test confirmed a statistically significant difference between the floodplain forest and the Schorndorf seed orchard in 2018 (p = 0.006). However, no significant difference was observed between the two seed plantations for 2020. In Schorndorf, seed viability was 159% higher in 2018 than in 2020, but different methods were used in the two years (Fig. 7). In addition, it should be noted that only

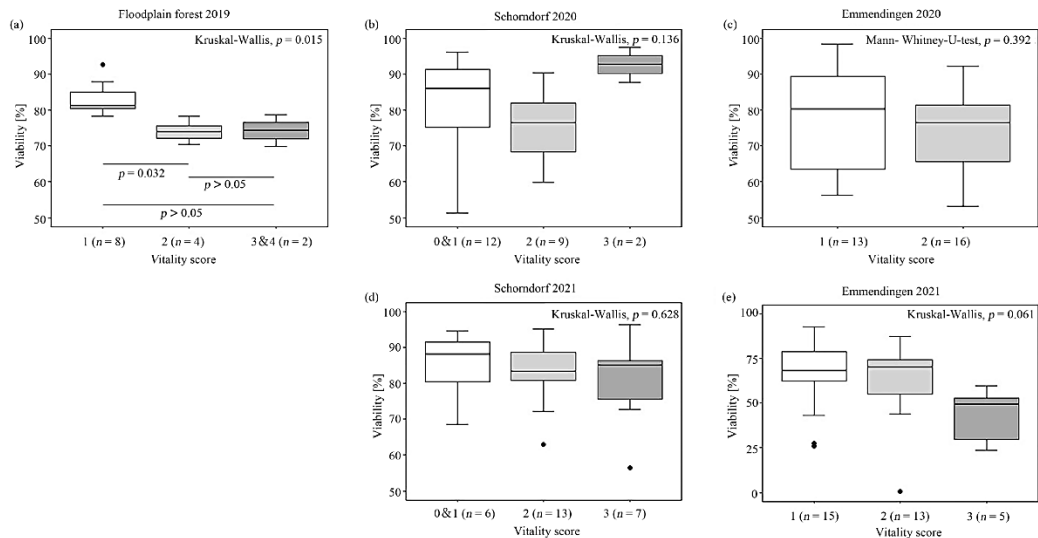


Fig. 5 Boxplots of pollen viability classified as healthy (0 and 1: white), diseased (2: light grey), and severely diseased trees (3 and 4: dark grey) for the floodplain forest, and the Schorndorf and Emmendingen sites in 2019, 2020, and 2021; interquartile range is represented

by the height of the boxes, maximum and minimum values by the upper and lower whiskers, the median by bold horizontal lines in the boxes, points indicate outliers. Numbers in brackets are the number of trees examined

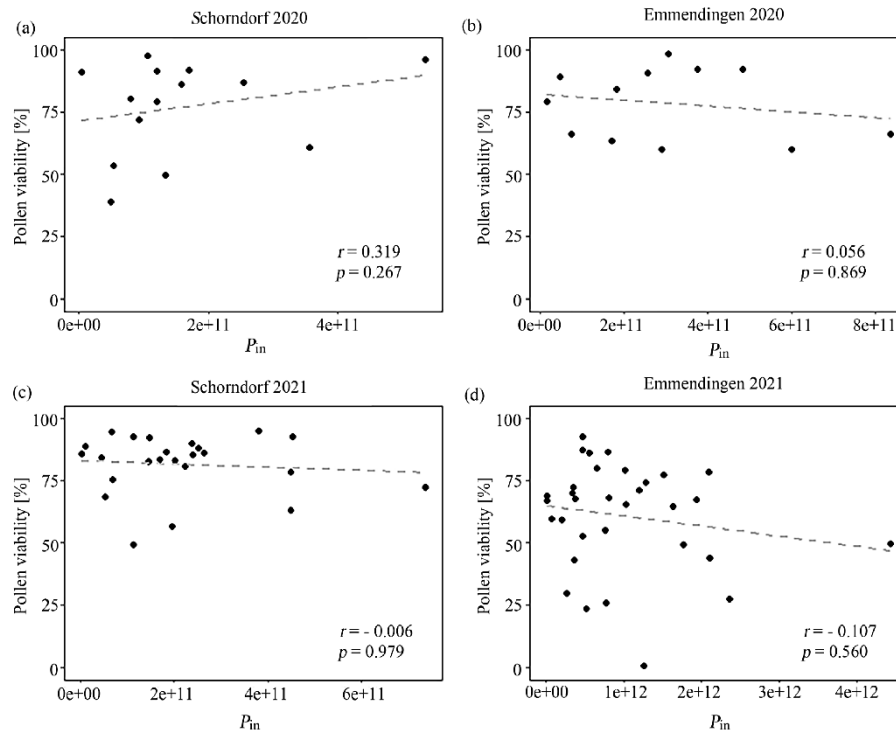


Fig. 6 Scatterplots with the variables pollen viability and pollen production per tree (P_{in}) for the Schorndorf and Emmendingen seed orchards in 2020 and 2021; the dashed line is the regression line, r the Spearman's correlation coefficient and p the significance value

seeds from mother trees with vitality values of 0, 1, and 2 were harvested in Schorndorf in 2018. Generally, seed quality was not statistically different between healthy and diseased trees ($p > 0.05$) (Fig. 8).

Discussion

Effects of ash dieback on flowers and pollen production

An important finding of our study was that only half of the male trees with severe crown damage produced inflorescences, substantially less compared to healthy trees. Additionally, it appeared that healthy trees had greater pollen viability. This is consistent with the findings of an earlier study in which paternity analysis was used to show that severely damaged trees were linked to limited pollination success (Eisen et al. 2023). Semizer-Cuming et al. (2021) also found that ash dieback decreased individual reproductive success through genetic analysis and the application of a spatial mating model. However, our results also show

that inflorescences of damaged trees did not significantly yield less or more pollen than healthy trees. Therefore, with the available data, we were unable to confirm that infestations of the fungus *H. fraxineus* caused an increase in pollen as a result of stress conditions. However, it should be noted that the increase in pollen emissions documented by Gassner et al. (2019) occurred approximately two years after ash dieback was locally detected. Nevertheless, it can be assumed that ash dieback has been widespread in southern Germany for more than ten years (Schumacher et al. 2007; Enderle et al. 2017). Therefore, a possible explanation could be that pollen production of infected trees does not remain high over a lengthy period of time but increases considerably immediately after the onset of the disease. This is also supported by the fact that a limited inflorescence production was observed for heavily infected trees. In 2020, inflorescences were detected on only two trees in vitality class 3, and there were none in vitality class 4. In 2021, there were nine trees in vitality class 3, and five in class 4. Thus, the sample size of the study trees with different degrees of infestation varied

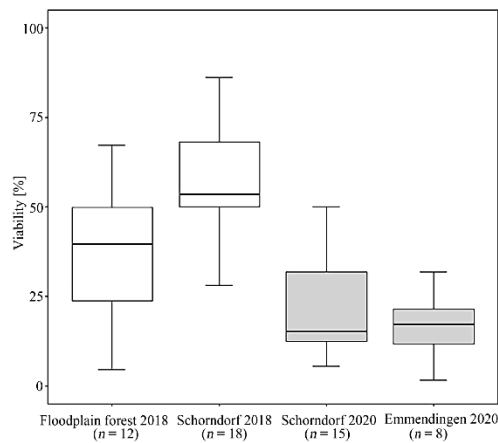


Fig. 7 Boxplots for germination or viability of seeds for the floodplain forest, and the Schorndorf and Emmendingen seed orchards in 2018 and 2020; n indicates the number of trees examined. Seeds from 2018 were stratified and germinated; seeds from 2020 were evaluated based on TTC staining. The interquartile range is represented by the height of the boxes, maximum and minimum values by the upper and lower whiskers, the median by bold horizontal lines in the boxes, points indicate outliers

considerably and should not be disregarded when interpreting the data.

Effect of locations on pollen production and quality

Environmental changes such as rising temperatures or increasing atmospheric carbon dioxide caused by climate change may influence pollen production and increase the reproductive potential (Bunce 2005; LaDeau and Clark 2006; D'Amato et al. 2007; Darbah et al. 2008; Zhang and Steiner 2022). Our estimated pollen production per anther was substantially low overall (10.8 cf. 27.7 thousand pollen grains) than that reported by Castifeiras et al. (2019) in Spain. The Emmendingen seed orchard, which is close to an industrial area and has a rather high average annual temperature for Germany, had a greater average pollen production per inflorescence (P_{inf}) than the Schorndorf seed orchard (Fig. 1). In contrast, the floodplain forest and the Schorndorf seed orchard, locations unaffected by direct impacts of road traffic, had the highest average viable pollen percentages. This is in agreement with other studies on pollen viability. Gottardini et al. (2004) and Duro et al. (2013) also found a negative correlation with air pollutants such as NO_x that have been linked to emissions from vehicle traffic. This can be reconciled by the fact that released, mature pollen are extremely dry and water-attracting and can absorb air moisture, including pollutants dispersed in the air (Comtois

1994; Duro et al. 2013). The pollutants can affect viability by triggering abnormalities in anthers, affecting germination and growth of pollen tubes and/or causing male sterility (Rezanejad 2007). However, the results obtained in this study on the average pollen viability of *F. excelsior* were higher (73% vs. 65%) than those obtained by Castifeiras et al. (2019) in Spain. This could possibly be explained by differences in climatic conditions of the study sites in Germany and Spain. According to Ge et al. (2011), the viability of *Panicum virgatum* L. pollen decreased five times faster under sunny than under cloudy conditions. Buchner et al. (2022) determined that environmental conditions were crucial to pollen viability and efficient pollination of female ash flowers. Through various climate chamber experiments, they showed an accelerated reduction in pollen viability with increased or prolonged UV radiation and under warmer conditions, whereas at low temperatures, viability could be maintained longer. Similar observations have been found in pollen studies of corn (Wang et al. 2010), oak (Schueler et al. 2005), and pine (Bohrerova et al. 2009). In addition, temperature is a crucial factor for the growth and development of flowers (Laaidi 2001). Varying temperatures that occur even within short distances (Jochner et al. 2011) can lead to asynchrony of flowering periods in ash populations which could result in pollination failure (Mondal et al. 2019). However, in addition to environmental changes, anthropogenic influences have a substantial impact on reproductive success. Crown pruning of individual ash trees at both seed orchards during harvesting operations to purposefully stress the trees, may increase pollen (Ranpal et al. 2022) and seed output. Another reason was to keep the trees short which makes it simpler to harvest propagation material (Bartsch and Röhrig 2016). Unfortunately, we did not have specific information regarding the treated trees and the extent of pruning. Otherwise, it would have been interesting to examine how treated and untreated trees differed in terms of pollen production and flower abundance.

Quality of seeds

There was not a statistically significant difference in seed quality between healthy and diseased trees. Several studies have investigated the presence of *H. fraxineus* DNA and RNA on seeds and shown that the pathogen was present on seed coats but not in necrotic or healthy embryos (Marčiulyrienė et al. 2018; van der Linde et al. 2021). In addition, the fungus could not be detected in axenically grown seedlings, which derived from seeds where the fungus was on the surface (Marčiulyrienė et al. 2018).

However, seed quality was generally very low in our study. Exclusively in the flower-rich year 2018, the germination capacity of the seeds in the Schorndorf orchard was above 50%. In addition, statistical analysis showed

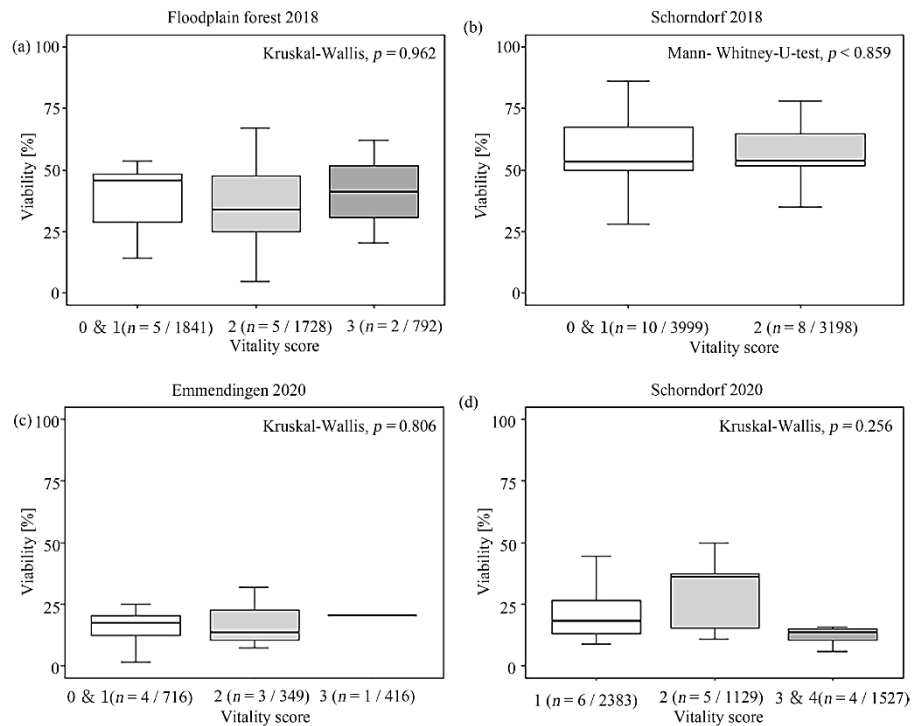


Fig. 8 Boxplots of seed quality divided into healthy (0 and 1: white), diseased (2: light grey) and severely diseased (3 and 4: dark grey) for the floodplain forest, and the Schorndorf and Emmendingen seed orchards in 2018 and 2020; interquartile range is represented by the

height of the boxes, maximum and minimum values by the upper and lower whiskers, the median by bold horizontal lines in the boxes, points indicate outliers. Numbers in brackets are the number of trees/seeds examined

that the mean viability by stratification was higher than those obtained with TTC. Generally, nurseries assume a seed germination rate of 58% to 65%, with only about 15 survivable seedlings expected per 100 seeds (Roloß and Pietzarka 1997; Schirmer 2002). Ash compensates for low germination rates by high seed production and can have regeneration rates of up to 150,000 individuals/ha under natural conditions (Tabari and Lust 1999). However, our lower than expected germination rates may indicate that ash dieback generally reduces seed quality because female trees invest less in reproduction due to higher reproductive costs (Cipollini and Whigham 1994; Antos and Allen 1999; Obeso 2002; Ueno et al. 2007). Thus, healthy ash trees may expend more energy on strengthening their immune systems, while diseased trees may need to focus efforts on minimizing crown damage rather than producing high quality seeds. In this context, Semizer-Cuming et al. (2019) also found that healthy female ash trees produced significantly more seeds than diseased trees, suggesting that female trees have

lower seed production due to crown damage. This damage requires resources during the growing season to restore the crown with new shoots and to produce reproductive tissue. This is particularly interesting as it is the opposite for male trees where there was no significant relationship for pollen production but for pollen viability.

In addition, it was found that all non-viable seeds were heavily infested with insects. This was possibly linked to the ash seed weevil (*Lignyodes* spp.), whose larvae develop and feed in ash seeds (Mwangola et al. 2022; Soldi et al. 2022). It is interesting that the ash seed weevil was particularly prevalent in the flower-rich year 2018, possibly because there were so many seeds available. This would increase the insect's food supply and enable it to thrive. However, since the insect does not distinguish between seeds from diseased and healthy trees, it can also influence gene flow from ash trees and reduce the effective dispersal of seeds (Mwangola et al. 2022).

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6.4 Pollen Viability of *Fraxinus excelsior* in Storage Experiments and Investigations on the Potential Effect of Long-Range Transport



Article

Pollen Viability of *Fraxinus excelsior* in Storage Experiments and Investigations on the Potential Effect of Long-Range Transport

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Abstract: Fragmented ash populations due to ash dieback may lead to a limited gene flow and pollination success. Therefore, the viability of ash pollen plays a major role for the survival of the species. The extent to which the long-distance transport of pollen affects pollen viability was investigated with experiments in a climate chamber using ash pollen samples from a seed orchard in Emmendingen, Germany. Furthermore, experiments with a volumetric pollen trap were conducted. A suitable storage temperature for ash pollen was determined by using four viability tests: TTC test, pollen germination, Alexander's stain and Acetocarmine. An optimization of the germination medium was performed. We found a strong influence of prevailing temperatures on pollen viability, which decreased faster under warmer conditions. At moderate temperatures, viable pollen could still be observed after 28 days. Thus, a possible successful pollination can also be associated to long-range transported pollen. Storage experiments showed that pollen viability could be maintained longer at temperatures of $-20\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$ than at $4\text{ }^{\circ}\text{C}$. In particular, the TTC test has proven to be suitable for determining viability. Therefore, properly stored pollen can be used for breeding programs to support the survival of *Fraxinus excelsior*.

Keywords: Acetocarmine; Alexander's stain; ash dieback; pollen germination; TTC; volumetric pollen trap



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1. Introduction

The common ash (*Fraxinus excelsior* L.) was formerly thought of as a suitable tree species for Europe's forests in the course of climate change [1,2]. Nowadays, this tree species is severely threatened by ash dieback, caused by the fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya [3]. Despite the current lack of effective conservation measures or opportunities to control ash dieback, there is hope for *Fraxinus excelsior* populations. According to the current literature [4–6], there is a natural variability in the susceptibility of ash trees to *H. fraxineus*. Different degrees of damage were attributed to a genetically determined resistance of ash tree individuals [7–9]. Less susceptible ash trees may have the potential to counter ash dieback through successful reproduction within breeding programs [10]. The proportion of ash trees that is (partially) resistant to ash dieback is estimated to be low, between 1% and 5% [11–14]. In addition, the natural regeneration of ash trees is believed to be of importance in fighting ash dieback since those seedlings, which are able to withstand competition, might constitute resistant phenotypes [15].

Within the next 30 years, an ash tree decline of 75% is expected in mixed stands in Europe [16]. A sharp decline in the ash populations is associated with stand fragmentation [13], probably resulting in the loss of genetic diversity and an increase in inbreeding

among ash individuals [17]. Studies of fragmented ash stands in Scotland have shown that gene flow between isolated ash trees can be maintained by pollen transport from distances of up to 2900 m [18,19]. However, it is known that pollen from anemophilous plants can be dispersed at mesoscales, i.e., 10–200 km from the source [20,21] and even synoptic scales [22,23]. These events are not frequent since they require specific environmental conditions including the characteristics of the landscape [19] and meteorological condition [24], however, they can be an important mean for gene flow providing that the pollen viability is maintained.

The success of atmospheric pollen transport depends crucially on pollen viability. At the same time, breeding programs with genetically more resistant ash trees can only be carried out successfully if viable pollen are available. The determination of pollen viability can be achieved using different methods. Besides the assessment of pollen germination, viability can also be studied via pollen staining tests [25,26]. The TTC test is a commonly used agent and proved successful determination of pollen viability for many species [27–31], although not all pollen species yielded successful staining [32,33]. For pollen of *Fraxinus excelsior*, this test was successfully applied by Castiñeiras et al. [34]. Furthermore, in our study viability was also tested using Acetocarmine, Alexander's stain and pollen germination. So far, no application of Alexander's stain has been documented for *Fraxinus excelsior*. For pollen germination, difficulties in achieving reliable results were reported [34]. In addition, no systematic optimization of the nutrient medium for pollen germination of ash pollen has been published as of yet.

We hypothesize that the viability of ash pollen decreases after their release from the flower and strongly depends on meteorological conditions during pollen flight. Thus, the aim of this study was to investigate pollen viability under different environmental conditions and to experimentally evaluate storage temperatures using different methods. In addition to climate chamber experiments that simulated the conditions pollen can face during atmospheric transport, the viability of pollen sampled from the atmosphere has been tested.

2. Materials and Methods

2.1. Study Material

For the experiments conducted in this study, ash pollen collected in an ash seed orchard were used. The seed orchard near Emmendingen (48°6'38.50" N, 7°52'20.49" E, 209 m NHN) is located in Baden-Württemberg, Germany. In 1995, the plantation was established on a previously agriculturally used area as a first afforestation on an area of about 2.7 ha. A total of 49 clones were planted, and in the summer of 2021 there were 84 ash trees on the plantation. The annual average temperature is 10.2 °C and the average precipitation sum is 882 mm (DWD station "Emmendingen-Mundingen", 1981–2010).

For the preparation of a pollen composite sample, shoots of male ash trees were cut shortly before flowering in March 2021. The shoots were then placed in vessels with water at room temperature in the laboratory. After approximately four days, the flowers of the shoots opened and pollen were released. These were then collected on paper underneath, mixed together and immediately used for the subsequent experiments.

2.2. Viability Tests

2.2.1. TTC Test

The TTC test is an enzymatic test and detects the presence of enzyme activity in pollen, according to which the viability of the pollen is determined. For this purpose, a colorless, water-soluble tetrazolium salt was used, which is reduced to red formazan in the presence of dehydrogenase in the pollen. The red colored pollen thus signals the viability and is only produced when there is active enzyme activity in the pollen [26]. To perform the TTC test, a 1% TTC solution of 0.2 g 2,3,5-triphenyl tetrazolium chloride and 12 g sucrose in 20 mL distilled water was produced. A thin layer of the pollen from the mixed sample was dusted with a brush onto a microscope slide. Afterwards, two drops of the TTC

solution were added to the slide and covered with a coverslip. The slide was then kept in a petri dish with moist filter paper. To achieve the maximum intensity of coloring, an incubation period of 24 h was set. After 24 h, the stained pollen were counted under a light microscope (Olympus CX23, magnification $\times 40$). Red stained pollen were categorized as “viable” and colorless or yellowish stained pollen were categorized as “non-viable”. We counted 400 pollen located on a random central row of the microscope slide. The evaluation of the TTC test was sometimes affected by a color gradient of the stained pollen, which made it difficult to distinguish clearly between viable and non-viable pollen. For this reason, we additionally included the classification “semi-viable” for pollen that were only colored slightly reddish. The coloration of pollen is often assessed very subjectively, and in particular, the boundaries according to which pollen are assigned to a certain category can vary depending on the processor [26]. Hence and in order to increase the accuracy, the microscope slides were only analyzed by one trained person in our study.

2.2.2. Alexander’s Stain

Malachite green, a component of Alexander’s stain is used to stain the cellulose of pollen walls green, while acid fuchsin and orange G are used to stain the cytoplasm pink. This test of pollen viability is based on the assumption that pollen containing cytoplasm can be regarded as viable [35]. Since Alexander’s stain contains toxic components, an adapted solution according to Peterson et al. [36] was used. The pollen were again finely spread on a microscope slide using a brush and then two drops of the solution were added. After covering the slide, it was heated on a hot plate for a few seconds until visible bubbles formed under the coverslip. Viable, semi-viable and non-viable pollen were counted after 10–15 min under the light microscope.

2.2.3. Acetocarmine

Acetocarmine stains existing chromosomes in the pollen [25]. Viable pollen are stained red, while non-viable pollen remain colorless. Again, when performing this test, pollen were first dusted onto a slide using a brush. Two drops of Acetocarmine were then applied to the slide and covered with a coverslip. The counting of pollen was conducted immediately after application using light microscopy.

2.2.4. Pollen Germination

Finally, the viability of the pollen was tested by pollen germination on a solid nutrient medium. For the storage experiments, a solution of 10% sucrose, 1% agar and 20 ppm boric acid in distilled water was used. After boiling the solution shortly, and allowing 14 mL to set in a 90 mm diameter petri dish, pollen were finely dusted onto the solid culture medium. Germination of the pollen then took place in the dark at room temperature for 24 h. After 24 h, pollen were counted under a light microscope and categorized as germinated or not germinated. Pollen were scored as germinated once the pollen tube was equal or larger than the diameter of the pollen grain [26]. As the germination values differed greatly from those of the other viability tests, an optimization of the culture medium was initiated.

For this purpose, a multi-stage experimental set-up was designed, according to which the ideal concentrations of the components of the culture medium for the pollen of *Fraxinus excelsior* were determined step by step. Different concentrations of sucrose, boric acid (H_3BO_3), calcium nitrate ($Ca(NO_3)_2$) and agar were investigated and three tests were conducted for each concentration. Five different culture media were prepared with different sucrose concentrations (0%, 5%, 10%, 15% and 20%). Since findings from our previous pollen germination experiments could be used, 20 ppm boric acid and 1% agar were also used for this experiment. The subsequent germination phase of 24 h took place in a regulated climate chamber (growth cabinet KBWF, Binder GmbH, Germany) at a constant temperature of 25 °C and 80% relative humidity in the dark. Then, the best sucrose concentration determined in the previous step was used and 20 ppm, 100 ppm, 200 ppm, 500 ppm and 1000 ppm boric acid were added to the prepared culture media with 1%

agar, respectively. The best concentration determined was further used in the third step of the optimization, which focused on calcium nitrate concentration. Calcium nitrate was investigated as an additional component of the nutrient medium, a substance that can often lead to a more successful pollen germination [37]. For this purpose, the concentrations 0 ppm, 100 ppm, 300 ppm, 500 ppm and 1000 ppm were tested. In the last step of the optimization, the concentration of agar was examined. Sucrose, boric acid and calcium nitrate concentrations, which were already determined, were used with 1% and 2% agar. Three repetitions were performed for each concentration for all components.

For the pollen viability derived from the different concentrations of sucrose, boric acid and calcium nitrate, a one-factor analysis of variance (ANOVA) and a subsequent post hoc analysis, the paired *t*-test, were performed in R (version 3.6.3). The suitability of the ANOVA was determined in advance by testing the variables for normal distribution (Shapiro–Wilk test) and variance homogeneity using Levene’s test. A two-sided *t*-test was used for the results of the two different agar concentrations.

Finally, a control with non-viable pollen was carried out according to Rodriguez-Riano and Dafni [38]. For this purpose, ash pollen were heated for approximately 15 minutes on a hot plate at 100 °C on a microscope slide. Afterwards, the non-viable pollen were tested for viability with the four viability tests, and thus, also for the validity of the tests used.

The TTC test, Alexander’s stain, Acetocarmine and pollen germination were used for the storage experiments, however, for experiments in the climate chamber and for the volumetric pollen trap, only the TTC test was used. For each experiment, we repeated each test three times.

2.3. Storage Experiments

Immediately after the preparation of the composite sample, the first viability tests were performed and the initial viability value was determined. Subsequently, the composite sample was stored at 4 °C in a fridge, at –20 °C in a common freezer and at –80 °C in a blast freezer (Fryka Cold box B 35-85). After one, two and three months, respectively, the viability of the samples was tested again. Please note, that results are not available for all investigated times due to delays in the delivery of individual chemicals and difficulties in carrying out pollen germination.

2.4. Experiments on Pollen Viability after Potential Atmospheric Transport

2.4.1. Climate Chamber

For the investigation of pollen viability under simulated natural conditions, three experiments were executed with different settings in a climate chamber. Each trial covered a period of 28 days. The viability of ash pollen was tested with the TTC test initially, after 24 h, 3 days, 7 days, 14 days, 21 days and 28 days.

For experiment 1 (Table 1), we firstly determined the mean April temperature and relative humidity for Bavaria since the flowering of ash predominately occurs in this month. Hourly data of 17 weather stations from the German Weather Service (DWD) (https://opendata.dwd.de/climate_environment/CDC/observations_germany/climate/hourly/ accessed on 15 November 2021) were averaged for the reference period 1961–1991. Data were divided into day (from 7 a.m. to 8 p.m.; 10 °C, 65% relative humidity) and night means (from 8 p.m. to 7 a.m.; 5 °C, 80% relative humidity).

Table 1. Climate chamber settings (temperature, relative humidity and UV radiation) for day and night conditions within experiments 1, 2 and 3 to test the influence of meteorology on the viability of ash pollen.

	Experiment 1		Experiment 2		Experiment 3	
	Day	Night	Day	Night	Day	Night
Temperature	10 °C	5 °C	12 °C	7 °C	20 °C	12 °C
Relative humidity	65%	80%	65%	80%	60%	80%
UV radiation	on	off	on	off	on	off

Experiment 2 represents a climate change scenario of +2 °C warming (Table 1), which can be expected according to the SSP2-4.5 scenario, by the middle of the 21st century for global means [39].

Experiment 3 investigated the influence of extreme temperatures on the viability of ash pollen with temperatures of 20 °C during the day and 12 °C during nighttime. These temperatures can obviously occur in April, although not constantly over a 28-day period. However, with these settings, the influence of higher temperatures can be studied in more detail.

The corresponding relative humidity for experiment 2 and 3 was estimated based on its relationship to the temperature using the hourly meteorological data of the DWD. UV radiation for all experiments was adjusted to the average potential sunshine duration prevailing in April (day 13 h) (Table 1).

2.4.2. Volumetric Pollen Trap

To investigate the viability of pollen under partially natural occurring transport conditions, samples of pollen suspended in the atmosphere during four weeks of the common ash pollen season in 2021 were collected using a volumetric pollen trap of the Hirst type [40]. Pollen were caught within the trap for a duration of seven days and for four weeks in total. The viability resulting from these data was assessed on a daily basis. Therefore, the pollen that were adhered at the last day were exposed to ambient air and the air inside the sampler either for a very short time (hours or minutes in the case of a quick impaction after pollen release) or probably also even longer when prior transport took place. In turn, the pollen of the first sampling day of one week was exposed to the influence of surrounding air for seven days within the trap, and probably for an additional time in the ambient air.

The sampling was performed on the roof of a university building in Ingolstadt (48°76′58.06″ N, 11°41′56.23″ E), Bavaria, at 13 m a. g. l. There are numerous ash pollen sources surrounding the sampler, as there are parks and green areas close to the pollen trap.

The drum of the pollen trap was changed weekly, starting on 15 April 2021. The silicone-coated foil strip attached to the drum was covered with a TTC solution shortly after each change and split into seven 48 mm long sections, each section corresponds to a 24 h sample, which was placed on the glass slide and examined under a light microscope at ×40 magnification. Ash pollen were checked for their viability along horizontal lines across the full range of the microscope slides and sorted into the categories of viable and non-viable until the number of 100 was reached. In total, on ten of 26 days the total count of all pollen on the slide was less than 100, due to a generally low pollen flight intensity on these days. The pollen concentration was calculated, after counting ash pollen of four random lines of the slide (representing approx. 9% of the impaction area), according to Galán et al. [41].

The air temperature was recorded throughout the study period, with a measuring device (Davis Vantage Pro 2) next to the pollen trap.

3. Results

3.1. Viability Tests

The applicability of the four viability tests for ash pollen was firstly evaluated on the basis of the quality related to distinction and recognition: The TTC test enabled a classification of the pollen into three categories (Figure 1a) based on the colors from dark red to pale yellow or colorless. Within the experiment of pollen germination, pollen tubes formed after 24 h, and could easily be recognized under the microscope (Figure 1b). Alexander's stain colored the pollen in clearly distinguishable pink and green colors (Figure 1c). In contrast to the TTC test and Alexander's stain, Acetocarmine led to a faint color, although viable and non-viable pollen could be differentiated based on pale reddish and colorless pollen. In addition to a coloration of the pollen, an increase in the size of the colored pollen was also observed after approx. 2 h when using Acetocarmine (Figure 1d), which decreased again after approx. 24 h.

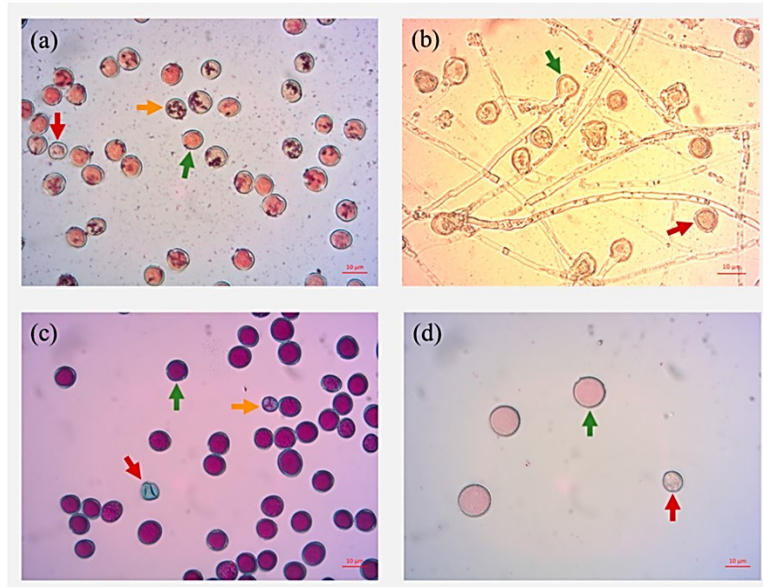


Figure 1. Exemplary pictures of the conducted viability tests, (a) TTC test, (b) Pollen germination, (c) Alexander's stain, (d) Acetocarmine. The green arrow points to exemplarily viable pollen, the yellow arrow to semi-viable pollen and the red arrow to non-viable pollen.

3.2. Optimization of Pollen Germination

For optimizing the nutrient medium for pollen germination, the ANOVA analysis revealed a significant difference between the five sucrose concentrations ($p = 0.020$). According to the post hoc analysis, there is no significant difference between 5% and 15%, and between 5% and 20%. The optimum sucrose concentration is 10%, which differs significantly to the other concentrations and was associated with the highest percentage of germinated pollen (Figure 2, Table 2).

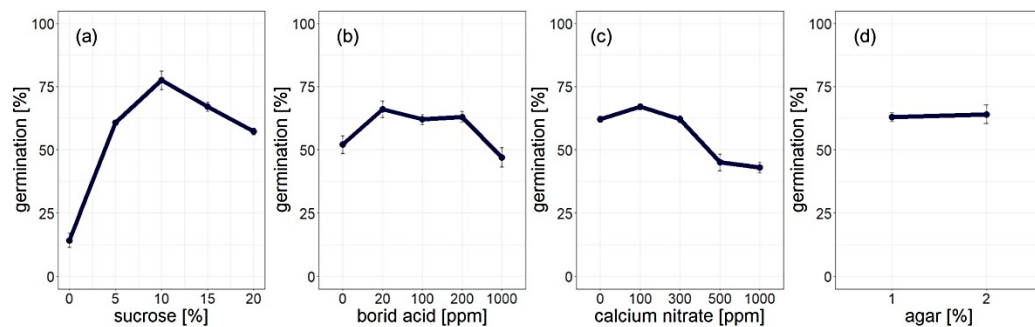


Figure 2. Results of the optimization of the procedure for testing ash pollen germination for different concentrations of sucrose (a), boric acid (b), calcium nitrate (c) and agar (d). The standard deviation is visualized with error bars.

Table 2. Results (*p* values) of the ANOVA post hoc paired *t*-test for comparing different concentrations of sucrose, boric acid, and calcium nitrate concentrations in the optimization of ash pollen germination.

		0%	5%	10%	15%
Sucrose	5%	<0.001 *			
	10%	<0.001 *	<0.001 *		
	15%	<0.001 *	0.084	0.005 *	
	20%	<0.001 *	0.131	<0.001 *	0.008 *
		0 ppm	20 ppm	100 ppm	200 ppm
Boric acid	20 ppm	0.008 *			
	100 ppm	0.059	0.554		
	200 ppm	0.034 *	0.694	0.694	
	1000 ppm	0.524	0.001 *	0.007 *	0.004 *
		0 ppm	100 ppm	300 ppm	500 ppm
Calcium nitrate	100 ppm	0.190			
	300 ppm	0.880	0.190		
	500 ppm	<0.001 *	<0.001 *	<0.001 *	
	1000 ppm	<0.001 *	<0.001 *	<0.001 *	0.720

* = significant difference with $p < 0.05$.

The optimal boric acid concentration was less clear. Even though the highest germination value was achieved at 20 ppm, the difference between 20 ppm, 100 ppm and 200 ppm was not statistically significant. Less suitable for pollen germination are 0 ppm and 1000 ppm. These groups were significantly different from the groups 20 ppm and 200 ppm and exhibited the lowest mean germination (Figure 2, Table 2). For the following tests, a concentration of 20 ppm was used.

For the calcium nitrate concentration, the highest germination value was observed at 100 ppm, however, this group was not significantly different from 0 ppm and 300 ppm. In particular, 500 ppm and 1000 ppm led to a lower germination of pollen (Figure 2, Table 2). For the following test, 100 ppm calcium nitrate were used.

Performing the two-tailed *t*-test on the two agar concentrations studied, no significant difference between the germination of pollen at 1% and 2% agar ($p = 0.913$) could be shown (Figure 2). For further tests 1% agar should be used.

Therefore, the optimum mixture yielding to the highest germination of ash pollen consists of 10% sucrose, 20 ppm boric acid, 100 ppm calcium nitrate and 1% agar, with calcium nitrate being an optional component.

3.3. Storage Experiments

The TTC tests showed that the viability of the pollen developed differently at the three investigated temperatures 4 °C, −20 °C and −80 °C after one, two and three months (Figure 3). In particular, the storage at 4 °C led to a more rapid decline in viability than at −20 °C and −80 °C and after two months, no viable pollen could be detected. The temperatures below freezing were associated with a decrease of 11.5% (−20 °C) and 6.7% (−80 °C) after three months.

Since the first test with pollen germination failed, the initial value is missing here. For pollen germination, pollen stored at −20 °C showed the highest viability values after one, two and three months. The viability of pollen stored at −80 °C was 11.9% lower. Pollen germination of pollen stored at 4 °C showed lower values in each month and no viable pollen from the second month onwards. In contrast to the expected decrease in pollen viability from the second to the third month, a slight increase ranging between 7% (−80 °C) and 12% (−20 °C) was recorded for all freezing temperatures (Figure 3). The documented increase can possibly be attributed to a higher room temperature and therefore a higher germination temperature during the incubation period (April vs. June).

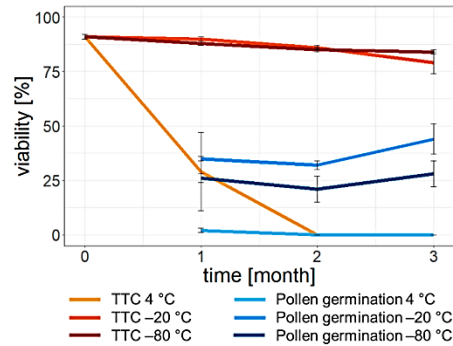


Figure 3. Temporal development of ash pollen viability [%] after one, two, and three months at storage temperatures of 4 °C, −20 °C and −80 °C evaluated with TTC test and pollen germination. The standard deviation is visualized with error bars.

Although pollen viability derived from the TTC test and from pollen germination differed, both tests showed changes in viability over the three months studied. The Alexander's stain and Acetocarmine test, however, did not present any change in the viability of the pollen. Even though not all values were available, it still was evident, that there were neither remarkable differences between the different storage temperatures nor differences between the individual months. The proportion of viable pollen in both tests was between 95% and 97% and thus higher than in the TTC test and pollen germination. Within the control test using heat-killed pollen, the TTC test and the pollen germination showed no staining or germination and thus proved their reliability. The Alexander's stain and Acetocarmine test on the other hand, yielded values between 95% and 97% of viable pollen in the staining of the killed pollen. These values are comparable to those from the storage experiments.

3.4. Atmospheric Transport

3.4.1. Simulations in Climate Chamber

The viability of the pollen exposed to the settings of climate chamber experiment 1 (10/5 °C, 65/80% relative humidity) was increased by 4% after the third day; then there was a decrease in viable pollen (−15%) after seven days. After 21 days, about half of the pollen were still viable, compared to the initial value. Even after 28 days, viable pollen (8%) could still be detected.

Experiment 2 differed by only +2 °C from the settings of experiment 1 (12/7 °C 65/80% relative humidity). An increase in the viability of ash pollen could be observed until the third day; after seven days the viability of the pollen decreased by 9%. On day 14, the viability of the pollen was only 10% below the documented initial value, and on day 21, it was only less than half of the initial value. After 28 days, 15% viable pollen could be detected.

The results of experiment 3 (20/12 °C, 60/80%) also show an increase in the viability until day three. At day seven, a rapid drop in the viability of the pollen was documented (−80%). After 14 days, no more viable pollen were observed under the microscope.

The comparison of experiments shows, that experiment 3 in particular differs from the two previous experiments (Figure 4). While viable pollen were still documented after 28 days in both experiment 1 and 2, no viable pollen were present in experiment 3 after only two weeks.

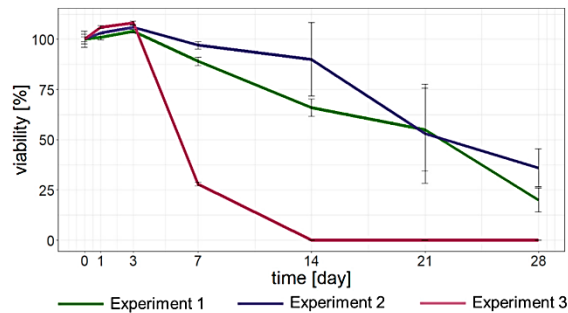


Figure 4. Temporal development of ash pollen viability [%] in the climate chamber for experiments 1 (10/5 °C, 65/80% relative humidity), 2 (12/7 °C 65/80% relative humidity) and 3 (20/12 °C, 60/80% relative humidity) within a total of 28 days. The standard deviation is visualized with error bars.

In all experiments, an increase in viability was observed up to day three. The initial viability value was determined using the TTC test, immediately after the pollen were removed from the blast freezer (−80 °C). These higher viability values can probably be explained by an increase in thawed pollen. This assumption is supported by the fact that in preliminary experiments, in which fresh pollen were used, no initial increase in viability was observed. However, it is also possible that the observed increases are due to counting inaccuracies, as the difference was only small (+4% to +8%).

3.4.2. Samples from Atmosphere

Within the pollen trap experiments, different viability values could be observed for the four investigated weeks. In week one, high viability values between 75% and 97% were recorded on all seven days (Figure 5). Lower viability values (17%) were recorded on day one in week two. That means that pollen from this day were captured in the interior of the pollen trap for at least seven days. On day seven, 62% viable pollen were sampled. That means that sampled pollen on this day were one day old or older in the case of long-range transported pollen. The mean pollen concentration was the highest of all four investigated weeks in week two and the aspired sample size of 100 ash pollen was achieved on all seven days. Viability values ranged between 17% and 90%. Week three is characterized by a low pollen concentration in the first half of the week and high viability values from day three onwards. In week four, only on one day 100 ash pollen could be counted. Since the number of counted ash pollen was low in week four, the calculated viability values are limited in their reliability. Overall, the viability of the pollen varied greatly between 0% and 97% over the course of the four weeks investigated (Figure 5). However, it can be seen that especially in week two (and week three), viability values were lower in the first days of sampling, i.e., for pollen that have a minimum age of seven days.

Meteorological conditions show lower air temperatures, especially in week one, and higher temperatures at the end of week four (Figure 5).

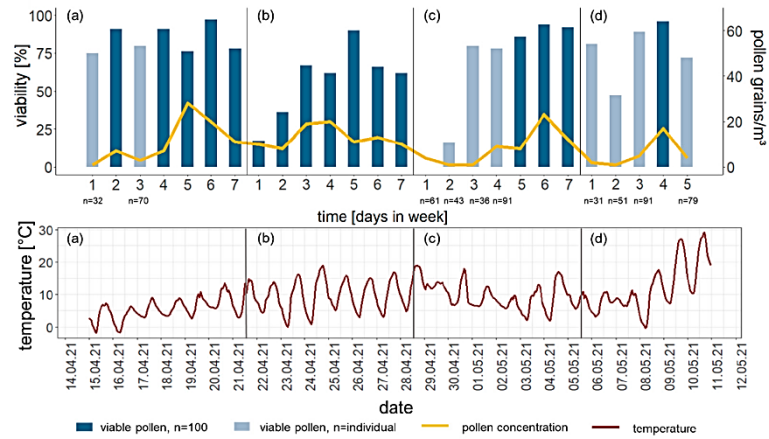


Figure 5. Viability of ash pollen [%] captured with a volumetric pollen trap (blue bars) in Ingolstadt, Germany in 2021, the respective pollen concentration [pollen grains/m³] (yellow line) and the ambient air temperature [°C] (red line) for week one (15.04–21.04) (a), two (22.04–28.04) (b), three (29.04–05.05) (c) and four (06.05–11.05) (d). Light blue bars indicate that the total pollen catch was <100 pollen grains (total numbers are shown below these bars), dark blue bars indicate that 100 pollen grains were counted and assessed for their viability.

4. Discussion

4.1. Suitability of Viability Tests for Ash Pollen

Knowledge on pollen viability is important with respect to increasingly fragmented ash populations and therefore an adequate assessment is crucial. The first test, the TTC test resulted in a successful staining of the pollen with a recognizable color differentiation. In this study, the validation using a control group of killed pollen [38] showed that no staining appeared. For ash pollen, the TTC test was already evaluated as a suitable test for determining their viability [34].

The Alexander's stain resulted in a clear coloring of the ash pollen in pink and green. While we are not aware of any study related to the pollen of *Fraxinus excelsior*, the Alexander's stain is frequently used for other pollen species. In particular, the clearly separated coloring of pollen is considered an advantage [42]. Good results with the Alexander's stain have been obtained, among others, in the assessment of the viability of pollen of *Polygala paniculata* L. [43], *Crotalaria juncea* L. [44], *Centaureum Hill* and *Gentianella Moench* [45]. The easy application of the stain, and the rapid results in particular, were rated as positive [46], which can be confirmed by our study using ash pollen.

The test is based on the assumption that the presence of pink stained cytoplasm simultaneously signals the viability of the pollen. While pollen containing no cytoplasm is certainly not viable, conversely, it does not necessarily mean that all pollen containing cytoplasm are viable. Therefore, pollen viability can easily be overestimated [47,48]. The results obtained in this study, which was based on adapted staining agents [46,49], also call this issue into question: Regardless of the duration of pollen storage and the respective temperature, we found that the average viability ranged between 95% and 97% and was considerably higher than that determined by the TTC test. In the control test with killed pollen, these pollen were stained just like viable pollen, which further confirms the unsuitability of Alexander's stain for ash pollen. The application of Alexander's stain for the determination of the development of the viability of *Nothofagus Blume* pollen under defined storage conditions also proved to be not successful [50]. This was also observed in *Asparagus officinalis* L. pollen after storage at 4 °C, while other viability tests resulted in a

change in viability. Storage conditions probably modify structures and processes within pollen, which affect the viability of these, however, this cannot be made visible using Alexander's stain [51].

Alexander's stain is suitable for studying the absence or presence of cytoplasm, and thus, the integrity of pollen or the proportion of sterile pollen [52]. For the ash trees, whose pollen were examined in this study, the percentage of sterile pollen ranged between 3% and 5%.

The staining of ash pollen with Acetocarmine provided a less clear staining than the TTC test or the Alexander's stain. Nevertheless, a differentiation between slightly reddish colored pollen and colorless pollen was possible. Good results were also obtained for pollen of *Colocasia esculenta* L. [53], *Lilium* Oriental hybrids [33] and *Ficus carica* L. [29]. In the case of *Castanea mollissima* Blume and *Castanea henryi* (Skan) Rehder and E. H. Wilson, it was not possible to distinguish between viable and non-viable pollen [32]. For *Olea europaea* L. pollen, a reliable assessment of pollen viability could also not be achieved, as shown by a killed control [54]. In this study, the control test showed that killed pollen were colored like viable pollen. Thus, the determination of the development of viability of ash pollen with Acetocarmine is not suitable. These results are consistent with the findings of Castiñeiras et al. [34], who reported that the staining of *Fraxinus excelsior* pollen with Acetocarmine provided high viability values close to 100%, as obtained in our study.

The composition of the nutrient medium used for pollen germination and the method of pollen germination varies greatly depending on the type of pollen examined. In general, both a liquid, as well as a solid culture medium, can be suitable. The nutrient medium of Brewbaker and Kwack [37] is often used, e.g., for determining the viability of *Svainsona formosa* (G. Don) Joy Thomps pollen [55], for *Acacia podalyriifolia* A. Cunn. ex G. Don and *Acacia mearnsii* De Wild pollen [56] or for *Elaeis guineensis* Jacq. pollen [57], however, individual optimizations were also frequently applied [58–60].

For the storage experiments, we used a solid nutrient medium with sucrose, boric acid and agar, and pollen germination was carried out at room temperature in the dark. Germination at room temperature has already been successfully performed for numerous pollen species, including pollen of various legumes (Fabaceae) [61] or pollen of *Svainsona formosa* [55]. A successful germination at room temperature was also achieved for pollen of *Fraxinus lanuginosa* Koidz [62]. In our study, we found that germination at room temperature was unsuitable. Although germination of the pollen was achieved and a difference between the different storage temperatures was observed, a comparison between the viability of pollen for different storage durations was impossible.

For this reason, a uniform temperature of 25 °C was selected for the subsequent optimization of the culture medium. According to Kremer and Jemrić [63], the optimal temperature for germination of *Fraxinus pennsylvanica* Marshall pollen is between 20 °C and 25 °C, with an optimum closer to 25 °C. Castiñeiras et al. [34] also used a temperature of 25 °C for *Fraxinus excelsior* pollen germination.

According to our results, the optimum sucrose concentration for *Fraxinus excelsior* pollen is 10%. For *Fraxinus lanuginosa* pollen, satisfactory germination was also achieved at 5% and 10% [62]. The highest germination values in the investigation of boric acid were found at 20 ppm, those of calcium nitrate at 100 ppm. Due to high viability values related to other concentrations, we conclude that a range of 20 ppm to 200 ppm for boric acid and a range of 0 ppm to 300 ppm for calcium nitrate is suitable for ash pollen analyses. Boric acid in particular represents an important component of the nutrient medium [25] and promotes the germination of *Fraxinus excelsior* pollen. The enhancement of pollen germination by the addition of boric acid has already been shown in other pollen species including *Litchi chinensis* Sonn [64] or *Cunninghamia lanceolata* (Lamb.) Hook [65]. The concentration of agar, however, did not reveal any remarkable differences between 1% and 2%.

Problems related to the performance of ash pollen germination were reported by Castiñeiras et al. [34]. Therefore, the optimizing tests of a solid nutrient medium performed in our study can be very helpful for future research on ash pollen.

Nevertheless, the evaluation of germinated pollen is associated with difficulties, as a strong germination with long, tangled pollen tubes can hinder the counting and thus the evaluation of the experiment. In addition, pollen germination is highly dependent on the environmental conditions of the experiment.

4.2. Effects of Storage Conditions on Ash Pollen

Breeding programs may benefit for the availability of viable ash pollen and therefore it is important to evaluate the optimal storage of pollen. The results of the Alexander's stain and the Acetocarmine test were not reliable in regards to the development of ash pollen viability under different storage conditions, the TTC test, however, provided good results. It was shown that a storage temperature of $-20\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$ is superior for preserving viability compared to $4\text{ }^{\circ}\text{C}$. Despite the limited evaluability of the results of pollen germination, pollen viability was also found to be least at $4\text{ }^{\circ}\text{C}$.

These results are consistent with studies on the viability of other pollen species under certain storage conditions. Pollen of strawberry have been shown to maintain their viability for 20 months at a storage temperature of $-18\text{ }^{\circ}\text{C}$, and for only eight months at $4\text{ }^{\circ}\text{C}$ [66]. *Phoenix Dactylifera* L. pollen also showed higher viability values when stored at $-20\text{ }^{\circ}\text{C}$ compared to $4\text{ }^{\circ}\text{C}$ [67]. Pollen viability of date palm was maintained for up to one year at $-20\text{ }^{\circ}\text{C}$ [68]. A higher viability was also observed in *Herbaceous peonies* pollen after storage at $-76\text{ }^{\circ}\text{C}$ compared to $4\text{ }^{\circ}\text{C}$ and $-20\text{ }^{\circ}\text{C}$ [69]. In contrast, other studies did not detect remarkable differences in pollen viability between storage at $-20\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$ (e.g., for almond pollen [70] or for *Leonurus cardiaca* L. pollen [71]). In these cases, it can be concluded that a storage at $-20\text{ }^{\circ}\text{C}$ is less expensive and therefore preferable. The observed slight decrease in germination of the frozen pollen in our study can probably be attributed to possible damage to the pollen due to the formation of intracellular ice during the freezing process, as also observed by Shekari et al. [71].

Our study covers only a storage duration of three months. Raquin et al. [72] found no decrease in viability of pollen from *Fraxinus excelsior* and *Fraxinus angustifolia* when stored at $-70\text{ }^{\circ}\text{C}$ for up to eleven months. Therefore, storage of frozen and viable ash pollen for longer than three months seems to be possible. This offers the opportunity to preserve and use ash pollen a long time after sampling for breeding programs. In the light of ash dieback, pollen of potentially resistant trees can successfully be stored and bear the potential for future breeding of trees that are able to cope with the disease.

4.3. Potential Effects of Long-Range Transport of Ash Pollen

We assume that long-range pollen dispersal affects pollen viability and we therefore evaluated the effects of meteorology on the quality of pollen in climate chamber experiments. We observed that pollen, which were distributed unevenly in the petri dish, probably led to shadow effects in the climate chamber experiments. That means that pollen that were located in the middle of a larger accumulation can be protected from the effects of UV radiation. In the first experiment, we were not aware of these shadow effects and from experiment two onwards, the pollen were then distributed in a very thin layer. However, due to the very small size of the individual pollen grains, shadow effects caused by surrounding pollen cannot be ruled out even in experiments two and three. Increased or prolonged UV radiation leading to a rapid reduction in pollen viability was demonstrated by studying maize [73,74], oak [75] and pine pollen [76]. To what extent, and in particular how quickly, the viability of the pollen examined is reduced differs greatly depending on the type of pollen. When interpreting our results, it has to be considered, that our experiments were designed to assume only cloudless days with a sunshine duration of 13 h. This is usually not the case for such a long period in the region (Emmendingen, Germany) from which we sampled the pollen. Under cloudy conditions, pollen viability of *Festuca arundinacea* Schreb. decreased more slowly [60]. For pollen of *Panicum virgatum* L., viability decreased five times faster under sunny compared to cloudy weather conditions [77].

The storage experiments have already shown that pollen viability can be maintained longer at lower temperatures. This was also confirmed by the climate chamber experiments. While the climate chamber experiments 1 and 2 with temperatures of 5/10 °C and 7/12 °C were associated with viable pollen that could still be observed after 28 days, in experiment 3 (12/20 °C) no viable pollen were present after only seven days. Experiments 1 and 2 differ from each other in the course of the development of viability, however, no clear conclusions can be drawn as to which of the two temperature settings ensures a longer maintenance of viability. Therefore, small changes in mean temperature were not associated with large effects on viability. However, long lasting heat extremes can have serious consequences for pollen viability.

The extent, and in particular, the time for which ash pollen maintains its viability after release thus depends on temperature (extremes) and the prevailing UV radiation.

The impact of natural environmental conditions on pollen viability was investigated in more detail by the volumetric pollen trap experiments. In particular, we determined whether a difference in viability could be detected between the days one to seven. Pollen sampled on day one may have been exposed to the prevailing temperatures and other meteorological conditions for six days longer. Since the foil band, on which the pollen were collected, was inside the pollen trap, the pollen already picked up were then protected from the influence of UV radiation. Meteorological conditions show lower temperatures at the beginning of the study period, however, a correlation with the viability values cannot be proven with the existing data.

In week two and three, lower viability values were documented for the first samplings days, which shows that pollen captured for a longer period may be negatively affected. With viable pollen ranging from 0% to 97%, viability of ash pollen differed greatly within the studied period. These data may present a rough proxy on the fraction of pollen, which was traveling a longer time before impaction.

A possible impact of the silicone adhered to the foil strip is unlikely, since we found a high viability of pollen on certain days.

5. Conclusions

We found that the duration of long-distance transport, and the environmental conditions prevailing at that time, play an important role in the successful pollination of female ash flowers by (long-range) transported pollen. In particular, high temperatures and sunny weather can have a negative effect on the pollination success since these meteorological factors reduce pollen viability.

Our methodological comparison showed that a successful pollen germination could be achieved using an optimized solid nutrient medium. Both Alexander's stain and Acetocarmine did not indicate the viability of the pollen correctly. However, the TTC test has proven to be suitable for determining viability, both in the storage experiments and in the long-distance transport investigations. The storage experiments have shown that storage of pollen at −20 °C or −80 °C is to be preferred to a storage temperature of 4 °C.

Overall, the viability of *Fraxinus excelsior* pollen in respect to ash dieback is shown to be of crucial importance. For future breeding programs using ash pollen, suitable methods to determine pollen viability are necessary, as well as an adequate storage preserving viability of the pollen. Natural reproduction of ash trees within and between prospectively fragmented ash populations is strongly dependent on pollen viability, and therefore, on the natural conditions during pollen dispersal. Future studies should focus on the influence of those natural conditions, especially UV radiation, however, they should also consider pollen production, which is potentially modified due to ash dieback.

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7 Summary and discussion of the main results

7.1 Ash pollen transport

Publications 1, 2 and 4 are devoted to the analysis of factors that may influence pollen transport in ash trees threatened by ash dieback. The studies provided information on the influence of meteorological factors such as temperature, precipitation and wind as well as the link to phenological development, stand structure and topography. However, it is important to distinguish between factors that directly influence the extent of pollen transport (i.e. wind speed or direction) and factors that indirectly influence pollen transport by affecting pollen release and concentration (i.e. temperature and precipitation). It will also be shown how the influence of these factors might affect the gene flow of the endangered ash tree and which measures might be needed to ensure a sufficient gene flow.

7.1.1 Indirect factors influencing pollen transport

Temperature

Temperature is a crucial factor for the onset of pollen release as it substantially influences flower growth and phenological development (Laaidi 2001; van Vliet et al. 2002; Jochner et al. 2011b; Hájková et al. 2020). This fact has also been shown in several studies analyzing long-term phenological time series (Menzel and Fabian 1999; Wielgolaski 1999; Jochner 2012). The results of Study 1 (Chapter 6.1) also showed that the onset of flowering was about one week earlier in 2020, the year, which was slightly warmer and drier in the spring than 2019 (Emmendingen: 26th March 2019 vs. 11th March, 2020; Schorndorf: 26th March 2019 vs. 18th March 2020). During field observations over the years, it was found that an increase in temperature stimulates pollen release, while a decrease in temperature can interrupt phenological development and pollen release. Thus, it can be assumed that different temperatures, which can occur even within small distances due to the topography (Jochner et al. 2011a) are linked to a certain spatial variability in phenological onset dates. The lack of synchrony in the onset of the pollen season potentially leads to pollination failure in a fragmented landscape (Eisen et al. 2022).

Furthermore, it was found that temperatures above 20 °C resulted in an increased pollen capture at the gravimetric traps (Eisen et al. 2022). Therefore, it can be assumed that higher temperatures result in increased anther dehiscence and a higher availability of pollen at this time point. However, those high spring temperatures may have both advantages and disadvantages for ash pollen transport: Since the reproductive success of ash depends on a sufficient amount of pollen reaching the female flowers, higher temperatures in spring may have a positive effect on the simultaneous release of many pollen (D'Amato et al. 2007; Fernández-Martínez et al. 2012; Zhang and Steiner 2022). This would also increase the probability of male pollen reaching female flowers. However, the influence of temperature on ash pollen production needs to be investigated in more detail using a longer time series in further studies. On the other hand, it was shown in Study 4 (Chapter 6.4) that the viability of airborne pollen decreases more rapidly under warmer conditions and with increased or prolonged UV radiation (Buchner et al. 2022). This could imply, especially for fragmented populations, that male pollen are exposed to such environmental conditions for a longer period of time due to the further distances they need to travel to the next female flower. Thus, these pollen may not be able to contribute to effective fertilization. In experiments conducted in a climate chamber at temperatures up to 20 °C (20/12 °C 60/80 % relative humidity), only 20 % viable pollen could be detected after 7 days and no viable pollen after 14 days. In contrast, at temperatures up to 12 °C (12/7 °C 65/80 % relative humidity), viable pollen could still be observed after 28 days (Buchner et al. 2022). Thus, it can be assumed that prolonged heat waves, which will be more common under climate change

conditions (IPCC 2023), may have serious consequences for pollen viability, especially during long-distance pollen transport.

Precipitation

Precipitation plays an important role in regulating pollen release and airborne pollen concentration (Laaidi 2001; D'Amato and Cecchi 2008) and thus indirectly influences pollen transport. D'Amato and Cecchi (2008) pointed out that dry conditions favor the release of pollen from the anthers and that high humidity or the occurrence of rain can interrupt this process. Our studies from Study 1 (Chapter 6.1) underlines that precipitation is linked to a decrease in pollen deposition (Eisen et al. 2022). Although light precipitation during our measurement campaigns had little effect on the amount of pollen recorded each week, high precipitation totals at the end of April 2019 and 2020 resulted in a substantial decrease in captured pollen at both orchards (Eisen et al. 2022). This suggests that precipitation may remove pollen particles from the atmosphere (Hughes et al. 2020; Schramm et al. 2021). However, it should be noted that the pollen data in our study were only available as weekly totals, and therefore a direct relationship with meteorological conditions, especially temporally fine-resolved precipitation events, could not be investigated. In addition, it was observed that the onset of rain at the end of March 2019 in Emmendingen was accompanied by an unexpected short-term increase in the amount of recorded pollen (Chapter 6.1; Eisen et al. 2022). This fact can probably be explained by the assumption of Šikoparija et al. (2018a): The impact of raindrops on a surface may create a vibration that leads to the release of pollen from anthers or from pollen already accumulated on surrounding leaves. In summary, while precipitation may have a short-term positive effect on airborne pollen concentrations by releasing pollen, it is more likely to cause a decrease in the longer term. Regarding ash gene flow, and when assuming greater distances between fragmented ash trees, an insufficient amount of male pollen could probably be transported to the female flower.

Phenology and synchronicity of flowering

In addition to temperature and precipitation, Study 1 (Chapter 6.1) showed that phenological onset dates are also a good indicator for inferring the timing and intensity of local pollen dispersal. This is due to the fact that the availability of flowers and their phenological development also indicate the presence of pollen. Overall, our phenological records agreed well with the periods reported in the literature (Albert et al. 2013; Douglas et al. 2013; Westergren et al. 2020). The main flowering phase (BBCH 65) started at the earliest on 20th of March (2020) in Emmendingen and on 1st of April (2020) in Schorndorf. The whole flowering period lasted for both orchards until 14th of May at the latest in 2019 (Eisen et al. 2022). Furthermore, most pollen at these sites were deposited in 2019 and 2020, while most of the surrounding male ash trees were in their main flowering phase (Eisen et al. 2022). On the one hand, the temporal correspondence between cumulative pollen trapping and our phenological observations suggests good synchrony among male flowers in the study areas, which could promote competition among male gametophytes (Bochenek and Eriksen 2011). On the other hand, it indicates that most of the trapped pollen were produced locally (Eisen et al. 2022).

In 2019, it was observed that flowers were often not fully developed and partially stunted. At the same time, gravimetric pollen traps recorded a pollen deposition, which was 11 times lower than in 2020 (Eisen et al. 2022). In the following years 2020 and 2021, however, a substantially higher number of flowers and a higher pollen load were detected (Chapters 6.1 and 6.3). This synchrony suggests a phenomenon also observed by Gassner et al. (2019), who found in every second to fourth year years with very low pollen concentrations followed by years with very high values in Switzerland. This suggests that there are not only mast years in seed production, but also in flower and pollen production. It is also interesting to note that pollen deposition at the pollen traps was observed as early as mid-March and therefore just before the locally and phenologically observed onset of pollen release (Eisen et al. 2022). This suggests that pollen reaching the pollen traps at this time originated

from outside the orchard (Estrella et al. 2006; Jochner et al. 2012). In turn, fertilization of ash trees in the orchard with pollen that has been transported over a longer distance seems possible.

The results of Study 2 (Chapter 6.2) also showed a foreign pollen input of more than 66 % at the Schorndorf seed orchard in the most year 2018 (Eisen et al. 2023). Since no other ash trees could be identified in the immediate vicinity of the orchard, it can be assumed that ash pollen from remote sources contributed to the fertilization. This also suggests long or medium distance pollen transport. Previous studies of effective pollen transport have shown that ash pollen transport in fragmented landscapes can occur over distances of up to 2.9 km, and that between 43 and 68 % of effective pollination is due to foreign pollen (Bacles and Ennos 2008). Again, this phenomenon can be attributed to the synchronization of flowering times and thus the phenology of ash trees (Mondal et al. 2019). For a successful fertilization, male and female flowers must open at approximately the same time. The flowering times of trees in a seed orchard may differ if parent trees are selected from different regions (provenances). If the flowering times of male and female flowers overlap only slightly, pollen from outside the orchard may have higher fertility at a given time (Douglas et al. 2013; Mondal et al. 2019). This external pollen input could affect seed quality and prevent the desired genetic exchange within the orchard. In fact, the local pollen cloud is thought to be sufficiently large in most years, which is one of the reasons why seed harvesting in orchards is usually limited to these years (Douglas et al. 2013).

In summary, phenological patterns and pollen availability of ash trees affect pollen transport only indirectly, but have a direct influence on gene flow. The temporal correspondence between flowering and pollen release, as well as variations in pollen loads, influence the likelihood of pollen transport and thus the exchange of genetic information between ash populations. As it is desirable for seed orchards that the majority of pollination occurs within the orchard, care should be taken when establishing future orchards. These should be designed as large areas with as many trees as possible to increase the likelihood of synchronous flowering between the genders.

7.1.2 Direct factors influencing pollen transport

Wind direction and topography

Wind, as a vectorial variable, is one of the main factors directly influencing pollen dispersal and the distance of transport in the air after pollen release (Laaidi 2001; Damialis et al. 2005; Puc 2012; Rojo et al. 2015a). However, the weekly resolution of the aerobiological data only allowed an analysis with respect to main wind directions. For other components, e.g., wind speed and persistence (Damialis et al. 2005), a finer temporal resolution of the data would have been necessary. Nevertheless, Study 1 confirmed that there is a link between wind direction and pollen count (Eisen et al. 2022): Especially in Emmendingen 2020, the studies showed that the prevailing wind direction often coincided with the direction from which most pollen were trapped (easterly and westerly directions). Semizer-Cumming et al. (2017) showed similar results and found a positive correlation between the prevailing wind direction and the mean direction of pollen dispersal in ash trees.

In contrast, a different phenomenon was observed in Schorndorf (Eisen et al. 2022): The slope and topography of the site favored the formation of cold air currents, in contrast to Emmendingen (open areas and no differences in elevation). Thus, it is possible that the released pollen were transported along the slope, especially during radiant nights, when cold air flows are likely to form. We recorded a particularly high amount of pollen at the base of the slope at the pollen trap furthest from the orchard (450 m from the center). In contrast, only very small amounts of pollen could be measured

on the upper slope in both years. In addition, the location of the climate station in Schorndorf was less favorable, as it was closer to the trees and not centrally located, in contrast to the station in Emmendingen. Thus, information on regional wind patterns was limited. In order to obtain more accurate results, we also included the nearby climate station of the German Weather Service (DWD) in Schorndorf (station "Kaisersbach-Cronhütte"), which led to a more precise agreement with the prevailing regional wind direction.

In addition, it was striking that the highest pollen catch outside the orchard was recorded in both orchards in 2019 (Eisen et al. 2022). According to Jato et al. (2006), pollen clouds are particularly abundant in the air when wind turbulence sustains pollen in the air or when weather conditions produce low wind speeds and transport pollen at very low speeds. Thus, depending on the turbulence at the time of release, pollen may have been transported upward and then away with the wind, only to descend again a few meters from the source (Skjøth et al. 2013; Adams-Groom et al. 2017). Thus, wind is influencing the presence of not only non-local but also long-distance transported pollen. Therefore, it is imperative that topography and prevailing dominant wind directions should be considered when establishing new seed orchards of resistant ashes in the future. As resistance in ash is highly heritable (Kjær et al. 2012; McKinney et al. 2014; Lobo et al. 2015), this can ensure that cross-pollination within the orchard contributes to the heritability of resistance and that high quality forest reproductive material is produced from less susceptible ash trees. In addition, the exact position of male and female ash plants should be aligned with the prevailing wind direction to increase the likelihood of pollination success.

Stand Structure and Pollen Availability

Another factor that affects pollen transport is stand structure. For example, Adams-Groom et al. (2017) found that pollen dispersal is more limited within a forest than in open areas. We can only partially agree with this finding: using the seed orchard in Emmendingen, we could indeed show in Study 1 (Chapter 6.1) that the dense edge planting on the eastern side of the orchard blocked pollen dispersal to some extent. The proportion of pollen caught in the traps directly to the rear of the orchard was less than 10 % of the total pollen catch. On the other hand, the highest pollen counts were measured at the outermost pollen traps in 2019 (Eisen et al. 2022), and pollen were also transported over distances of more than 550 m in the floodplain forest in 2018, despite the dense tree cover, and successfully contributed to pollination (Eisen et al. 2023).

This could be due to the height and canopy width of the pollen source (Adams-Groom et al. 2017). In the low pollen year 2019, we found that the few flowers that did develop were mainly in the upper parts of the crown (Eisen et al. 2022). This suggests that ash pollen were transported across the boundary planting and pollen traps placed close to the planting, and were deposited after a certain distance. Due to their small diameter of about 20-26 μm and their circular to triangular convex shape (Fægri 2000), ash pollen can be lifted and transported even at low wind speeds. However, in 2020, the amount of pollen recorded in the traps furthest away from the orchard was much lower than in the traps within the orchard. Thus, it cannot be excluded that pollen from neighboring ash trees contributed to higher levels at the edge of the study area. This could be the case especially for the furthest pollen trap in the east of the Emmendingen orchard. There, further ash trees were found in a wooded area to the north-east of the trap in the prevailing wind direction (the third most frequent during the campaign) (Eisen et al. 2022). However, the fact that an effective longer distance pollen transport was also observed in the floodplain forest (Eisen et al. 2023), which is dominated by mature old ash trees with canopy heights of up to 30 m, supports the first theory that ash pollen are initially transported several meters before being deposited, depending on canopy height. Furthermore, wind

flows accompanied by local thermal circulation can be responsible for a high pollen flow on the day of pollen release (Prtenjak et al. 2012; Negral et al. 2021).

For detailed investigations, additional pollen traps were set up at 5 m height in the Emmendingen seed orchard and were therefore closer to the pollen source (Study 1; Chapter 6.1). The average height of the ash trees was 12 m. Contrary to our expectations, our results showed that deposition totals did not differ significantly, although we expected to measure higher averages in the higher elevated traps. However, even though our data only provide weekly averages, we found temporal differences. The highest value was initially reached within the orchard in the higher traps, but outside the orchard it was reached during the final week of the surveys (Eisen et al. 2022). This suggests that pollen availability and pollination efficiency could be different at different heights.

Another relevant factor is the density of ash trees in the stand itself. The high level of foreign pollen input found in Study 2 (Chapter 6.2) at the Schorndorf seed orchard (Eisen et al. 2023) indicates that ash mortality probably has already affected pollination. The dead ash trees lead to increased tree spacing. This, in turn, may have led to an increasingly mixed pollen cloud and a higher proportion of pollen coming from outside to pollinate the flowers. Pollen dispersal patterns observed from paternity analyses support this hypothesis: trees at marginal areas of the seed orchard were found to have greater external pollen input than mother trees in the center of the orchard (Eisen et al. 2023).

In summary, for seed orchards it is recommended to collect seeds from central trees since there is a higher probability that both parents originate from the orchard. The studies also showed that it is quite reasonable to establish marginal plantings at the edges of the orchard to reduce pollen input from outside and to avoid cross-pollination. Conifers such as spruce are particularly suitable for this purpose. Deciduous trees or shrubs can also reduce pollen entry, but are less effective due to their more permeable structure. Deciduous trees often begin to leaf out after the ash has flowered, and shrubs can be disadvantaged by their low height. In natural stands, however, attention should be paid to open spaces and pathways due to fragmentation, which can also ensure gene flow between populations through pollen transport. Dense stands of spruce between populations could be counterproductive to effective pollen transport in this case.

7.1.3 Estimation of pollen transport distances

Using aerobiological measurements (Study 1; Chapter 6.1), we found that ash pollen were transported more than 400 m (Eisen et al. 2022). We were able to prove the same with the effective pollen transport studies (Study 2; Chapter 6.2), which showed that in the floodplain forest only 26.1% of the pollen could be traced back to their fathers, which means that pollen were transported over distances of more than 550 m and successfully contributed to fertilization (Eisen et al. 2023). This shows that the distance to seed orchards recommended by the German Forest Reproduction Act (FOVG 2002; BGBl. I p. 1658) does not prevent foreign entry.

However, the two studies also showed that the amount of pollen and pollination success decreased substantially with increasing distance. Study 1 (Section 6.1) showed that the amount of pollen in the air is already reduced by 50 % at a distance of 200 m from the source. At a distance of 500 m, it is reduced by about 80 % to 90 % (Eisen et al. 2022). In Study 2 (Chapter 6.2), the highest pollination rate of 70 % was observed in the orchard at a radius of 100 m, after which it decreased considerably. More than 50 % of the examined ash trees achieved fertilization within a radius of 140 m in the

floodplain forest, while only 8 % of the mother trees were located at distances greater than 400 m from the father trees. Overall, the average distance between father and mother trees was 76 m in the seed orchard and 166 m in the floodplain forest (Eisen et al. 2023). The results of both studies are very consistent with previous studies of effective pollen transport: for example, Semizer-Cuming et al. (2021) reported that 50 % of ash pollen dispersed within 140 m and only 5 % of pollen dispersed more than 1.3 km. Heuertz et al. (2003) reported values of effective pollen transport between 70 and 140 m.

As a measure to increase pollination within the orchard, it should therefore be ensured that the distance to other ash trees is substantially greater than 400 m. However, with regard to ash dieback, effective pollen dispersal over longer distances can be regarded as positive, as it can connect fragmented ash populations. The demonstrated genetic exchange also allows isolated ash trees that are less susceptible to *H. fraxineus* to pass on their genes and increase genetic diversity. In the long run, the genotypes that are adapted to the fungus and have the highest fitness under the prevailing environmental conditions may prevail, substantially increasing the chances of a healthier next generation.

7.2 Relationship between reproductive success and ash dieback

Publications 2 and 3 dealt with the effects of ash dieback on flower and pollen production, pollen and seed quality, and effective pollination success. Publication 4 supported these studies with experimental trials on pollen viability. The results of the studies indicate that ash dieback affects flower production, pollen viability and pollination success, but not pollen production and seed quality.

7.2.1 Effects of ash dieback on flowers and pollen production

Chapter 2.2.1 demonstrated that flower and pollen production play a key role in the transmission of genetic information to the next generation. This can be explained by the fact that successful pollination requires large amounts of pollen, and therefore interannual variation in pollen production directly affects seed production (Satake and Iwasa 2002; LaDeau and Clark 2006; Mangla and Gupta 2015). From this point of view, our phenological observations showed an overall positive outcome: Only half of the male ash trees with severe crown damage produced inflorescences, which was significantly less than for healthy ash trees (Chapter 6.3; Eisen et al. 2024). In 2020 and 2021, inflorescences were observed only in a maximum of nine ash trees in vitality class 3 and in five ash trees in vitality class 4 in the two plantations (Eisen et al. 2024). Thus, heavily infested ash trees do not pass on their genes to the next generation and contribute less to fruiting. This also reflects the results of Study 2, where we used paternity analyses to show that severely damaged ash trees have lower pollination success (Eisen et al. 2023). Similarly, Semizer-Cuming et al. (2021) found that ash dieback reduced individual reproductive success of ash trees through genetic analyses and the application of a spatial mating model.

However, our results from Study 3 showed no significant relationship between the health status of male ash trees and pollen production per flower as well as per tree. Consequently, based on the available data, the suggestion by Gassner et al. (2019) could not be confirmed. They assumed that infection with the invasive fungus *Hymenoscyphus fraxineus* could increase pollen emission, at least temporarily, because stress symptoms could lead to increased flowering in affected trees. However,

it should be considered that the increase in ash pollen emissions observed by Gassner et al. (2019) occurred approximately two years after the local occurrence of ash dieback. As ash dieback has been widespread in Southern Germany for more than a decade (Schumacher et al. 2007; Enderle et al. 2017), a possible explanation could be that pollen production of affected ash trees increases only immediately after the disease outbreak, but does not remain at a high level for a longer period of time. This hypothesis is supported by the evidence of low flower production in severely damaged ash trees.

Overall, our estimated pollen production per anther (10.77 thousand ash pollen grains) was substantially lower than the 27.66 thousand pollen grains reported by Castiñeiras et al. (2019) in Spain. However, it was noticeable that the Emmendingen orchard had a higher average pollen production per inflorescence (P_{inf}) than the Schorndorf orchard. This could be due to the location of the study sites: The orchard in Emmendingen, which experiences above-average temperatures and is located next to an industrial area differs from the orchard in Schorndorf, which is located in a forest clearing. Several studies have already shown that increasing temperatures and atmospheric carbon dioxide levels can influence pollen production and therefore reproductive potential (Bunce 2005; LaDeau and Clark 2006; D'Amato et al. 2007; Darbah et al. 2008; Zhang and Steiner 2022). Tree management can also strongly affect reproductive potential (Bartsch and Röhrig 2016; Ranpal et al. 2022). To expose ash trees to stress and specifically stimulate pollen and seed production, crown pruning was performed on individual ash trees in both seed orchards during harvest. Since it has not been documented in the past, which ash trees were pruned, no further investigations could be carried out in this context.

7.2.2 Effects of ash dieback on pollen quality

The studies on pollen quality showed a tendency that healthy ash trees were linked to a higher pollen viability. For example, a statistically significant difference in ash pollen viability was found for the floodplain forest in 2019, and marginal significance was also found for Emmendingen in 2021 (Eisen et al. 2024). As with pollen production, this can also be supported by the second study (Chapter 6.2), in which we showed that healthy ash trees were associated to a higher pollination success (Eisen et al. 2023). This has two positive implications: On the one hand, this contributes to natural selection by ensuring that the most resistant ash trees pass on their genes to the next generation, and on the other hand, the pollen from healthy ash trees can be used for conservation and breeding programs.

In this context, breeding programs can benefit from the availability of viable ash pollen. Therefore, in Study 4 (Chapter 6.4), we conducted experimental trials to determine the optimal storage conditions for ash pollen. The analyses showed that storage temperatures of -20 °C and -80 °C were very suitable for maintaining viability. Other studies also found no significant differences in pollen viability between storage at -20 °C and -80 °C (Martínez-Gómez et al. 2002; Shekari et al. 2016). In contrast, storage in a refrigerator showed little success (Buchner et al. 2022). This is also reflected in other studies investigating strawberry pollen (Aslantaş and Pirlak 2013), *Phoenix Dactylifera* L. pollen (Mesnoua et al. 2018; El Kadri and Ben Mimoun 2020) or peony pollen (Du et al. 2019). Overall, the storage period of Study 4 was only lasting for 3 months, but since Raquin et al. (2002) found no decrease in viability of *Fraxinus excelsior* and *Fraxinus angustifolia* pollen even after 11 months, this offers great potential for successful deposition and preservation of pollen from resistant ash trees for future breeding programs (Buchner et al. 2022).

However, the results on the average viability of *Fraxinus excelsior* pollen obtained in Study 3 (Chapter 6.3), with an average viability of 73 %, were higher than those obtained by Castiñeiras et al. (2019), with 65 % in Spain (Eisen et al. 2024). This difference in viability can potentially be attributed to the varying climatic conditions in the respective provenances, as discussed in Chapter 7.1.1 (Temperature). Thus, pollen viability tends to decrease more rapidly under warmer conditions and increased or prolonged UV radiation (Study 4; Chapter 6.4).

For example, Ge et al. (2011) found that the viability of *Panicum virgatum* L. pollen decreased five times faster under sunny conditions than under cloudy conditions. In addition, the study sites floodplain forest and Schorndorf had the highest average percentages of viable pollen. Both sites are not directly affected by road traffic, which favors pollen viability (Gottardini et al. 2004; Duro et al. 2013). This is due to the fact that released mature pollen are extremely dry and water-attracting and can absorb atmospheric moisture, including airborne pollutants such as NO_x (Comtois 1994; Duro et al. 2013). Viability can be affected by pollutants, for example, by causing abnormalities in the anthers, affecting pollen tube germination and growth, or causing male sterility (Rezanejad 2007).

7.2.3 Effects of ash dieback on seed quality

The results related to seed quality from Study 3 (Chapter 6.3) showed no statistically significant difference between healthy and diseased ash trees (Eisen et al. 2024). Thus, the disease occurrence has no direct effect on the quality of seed produced. In turn, Semizer-Cuming et al. (2019) found that healthy female ash trees produced substantially more seeds than diseased ash trees (Chapter 2.2.2). This is particularly interesting since it is exactly the opposite compared to male ash, where no significant correlation was found for pollen production, but for pollen viability. This may be due to gender differences in reproductive investment (Cipollini and Whigham 1994; Antos and Allen 1999; Obeso 2002; Ueno et al. 2007). For example, severely diseased male ash trees that still invested in flower and pollen production tended to rely on quantity rather than quality. Severely diseased female ash trees reduce the quantity of seed and are more likely to spend their resources on reducing crown damage (Semizer-Cuming et al. 2019) and probably the seed quality, as it is as good or as poor as in healthy ash trees. However, whether this theory is actually true should be analyzed in more detail in further studies.

In general, the seed quality was very low. Only in the year 2018, which was characterized by a high number of flowers, 57 % of the seeds from Schorndorf and 38 % of the seeds from the floodplain forest were able to germinate. In 2020, the mean viability values with the TTC test were even below 25 %. Therefore, seed stratification generally yielded higher mean viability values compared to the staining test (Eisen et al. 2024). However, since nurseries assume a germination rate of about 58 % to 65 % prior to ash dieback (Roloff and Pietzarka 1997; Schirmer 2002), this may indicate that disease outbreaks generally reduce seed quality. Thus, healthy ash trees must expend more energy to strengthen their immune systems, whereas diseased ash trees must focus their efforts on minimizing crown damage.

We also found that all non-viable seeds were heavily infested with insects (Eisen et al. 2024). This infestation is likely due to the ash seed weevil (*Lignyodes* spp.), whose larvae develop in and feed on ash seeds (Mwangola et al. 2022; Soldi et al. 2022). Interestingly, the ash seed weevil was particularly abundant in 2018, as this year was characterized by a high number of flowers, likely due to the large amount of available seeds. This increases the food supply for the beetle and allows it to develop well. However, because the beetle does not discriminate between seeds from healthy and

diseased ash trees, it could also actively affect gene flow from ash trees and reduce the effective dispersal of ash seeds (Mwangola et al. 2022).

7.2.3 Impact of ash dieback on mating success

Beside the direct effects of ash dieback on reproductive ecology, Study 2 (Chapter 6.2) also examined actual pollination success as a function of ash health status. The results of the study showed that healthy and slightly damaged male ash trees generally had the highest pollination success rate. In contrast, much lower pollination success was observed in severely damaged father trees (Eisen et al. 2023). These results are consistent with the findings of Study 1 (Chapter 6.1) and 3 (Chapter 6.3), in which it was shown that healthier male ash trees produced more flowers as well as pollen with higher viability. Thus, it can be assumed that crown damage caused by ash dieback reduces individual reproductive success (Semizer-Cuming et al. 2021). Accordingly, Semizer-Cuming et al. (2019; 2021) also found that ash trees with lower susceptibility to ash dieback had higher reproductive capacity compared to severely diseased ash trees.

However, it is likely that even severely damaged ash trees produced offspring, although to a much lesser extent. Some of the father trees in the floodplain forest were removed due to thinning measures prior to sampling and vitality assessment, and existed only as stumps. Due to the removal of the ash trees, it can be assumed that they had already been severely damaged by the fungus (vitality score > 2). Furthermore, in the seed orchard Schorndorf, only the average vitality classes of the clones were considered for the paternity analyses. As the ramets have identical genotypes per clone, the software cannot identify the actual parent tree. Therefore, the average vitality score was used, although individual ramets could have been characterized by different vitality scores (Chapter 6.2; Eisen et al. 2023). However, it is quite positive that self-pollination does not seem to have any influence on the reproduction of ash (Eisen et al. 2023). For example, in Study 2 (Section 6.2), self-pollination was detected with high confidence in only one offspring in the floodplain forest (Eisen et al. 2023). In addition, Saumitou-Laprade et al. (2018) suggest that ash trees are self-incompatible and that self-pollinated seeds are not viable due to inbreeding depression.

In summary, it can be deduced from the results that mainly less susceptible and slightly damaged ash trees successfully contributed to pollination. In contrast, heavily affected ash trees did not have a large number of inflorescences and thus did not contribute considerably to fruiting. This suggests that the diseased trees will not pass their genes to the next generation to a great extent, which is why this positive circumstance could possibly be a promising indication of the species' adaptive potential.

As disease resistance can be partially inherited from parents to offspring (Kjær et al. 2012; McKinney et al. 2014; Lobo et al. 2015), this could lead to a long-term reduction in the negative effects of ash dieback on the ash gene pool. However, this requires that a sufficient number of healthy or less susceptible ash trees with high genetic variation are maintained and successfully reproduce. In this way, future generations of ash trees that emerge through natural regeneration and can withstand competition within or between species may be less susceptible to ash dieback. These resistant ash trees would then be the product of natural selection and could potentially provide mechanisms for disease control (Metzler et al. 2012; McKinney et al. 2014; Jochner-Oette et al. 2021). Considering these findings, it is important that thinning measures in ash stands preserve healthy trees with little damage in order to maintain genetic diversity. This provides the foundation for the development of future resistant ash populations. At the same time, targeted breeding and planting of ash trees considered less susceptible could enrich the gene pool of existing forests and improve the resilience of ash populations (McKinney et al. 2014; Enderle et al. 2015).

8 Conclusions

Ash dieback is associated to far-reaching consequences for *Fraxinus excelsior* in Europe and, based on current knowledge, will continue to progress in the coming years. Nevertheless, the studies provide hope for the future of the ash tree, since it has been shown that as health deteriorates, reproductive capacity declines. In addition, it has been shown that healthy and slightly diseased father trees contribute more to pollination, implying that high vigor has a beneficial effect on reproductive success. Thus, in the long run, these ash dieback effects may result in ash populations that are less prone to ash dieback in the future, and less sensitive ash trees will predominate in regeneration. In contrast, severely damaged ash trees are less fit and may slowly disappear from the population due to reduced flowering intensity and pollen quality. This means that a healthier next generation of ash trees may emerge in the future.

Nevertheless, the obtained results suggest that a variety of other factors influence the reproductive ecology of ash, especially under conditions of climate change (e.g., heat waves, heavy rain events) (Ohnishi et al. 2010; Hedhly 2011; Carpenedo et al. 2017; Talwar et al. 2022) and increasing levels of atmospheric air pollutants (LaDeau and Clark 2006; Darbah et al. 2008; Duro et al. 2013). These extremes may also lead to novel disease outbreaks and damage to ash in the future. Therefore, the mechanisms of adaptability and disease resistance of ash need to be better understood. For this purpose, artificial crossing experiments could be performed under various conditions and monitoring of the bred ash trees established.

Continued fragmentation of ash populations will also lead to a decrease in opportunities for effective pollination. The results of this study have shown that effective pollen transport occurs over long distances, but with decreasing pollination success. Thus, the aerobiological studies also showed that ash pollen were transported over a distance of more than 400 m, but at a distance of 500 m only 10-20 % of the pollen could be captured. In addition, the results of the study showed a relation with pollen transport and meteorology, the onset of phenological development and the topography of the orchard. However, because the pollen data available in our study were only weekly totals, future studies should consider finer temporal resolution and more detailed measurements at different heights to better understand ash pollen dispersal.

In addition, the health status of ash trees is currently assessed solely visually using a six-level vitality score (Lenz et al. 2012a; Peters et al. 2021). However, this assessment requires experience and is frequently challenging because ash trees in natural stands can grow quite tall and the canopy is often obscured by understory. The application of remote sensing technologies allows information to be acquired quickly and repeatedly over a large spatial area. Therefore, this could be an alternative and effective additional method for detecting crown damage and monitoring disease in the future (Gašpa- rović et al. 2023).

However, this study clearly illustrates the critical importance of supporting the conservation of this valuable tree species and taking steps to promote its reintroduction into our forests. Although further research is needed, there is no doubt that ash trees play a substantial role – ecologically, economically, and culturally – in our society. Therefore, it is of great importance to secure its future and to take appropriate measures to strengthen its presence. Overall, the results of this study provide important indications of the adaptive potential of ash and underline its survivability in European ecosystems. Thus, important findings for the successful conservation of the species and for the handling of the ashes in practice could be derived from the results. This includes collecting grafts and seeds from healthy trees and establishing seed orchards and gene reserves. Despite the increasing fragmentation of ash populations, these actions can help to maintain broad genetic diversity and to

ensure the long-term viability of ash trees. Maintaining genetic diversity is not only critical to the survival of the species, but also increases its ability to adapt and evolve to future changes in the environment.

Besides the recommendations mentioned above, which are directly linked to the results of this PhD thesis, there are also other recommendations that already exist (Lenz et al. 2012b; Metzler et al. 2013; Fussi 2020): Thus, private forest owners can also contribute to the maintenance and promotion of natural resistance mechanisms by using collected seeds of less susceptible ash trees for planting young trees and supporting the growth of the dominant natural regeneration (Lenz et al. 2012b). Unless ash trees pose an immediate threat due to their reduced stability, it is advisable not to harvest even infested or dead ash trees, as many species depend on the dead wood (Lenz et al. 2012b; Metzler et al. 2013; Hultberga et al. 2020). To alleviate forest owners' concerns about complete failure, additional broadleaf species should be planted in the affected areas. Such mixed stands not only meet different needs, but also show greater resistance to invasive pathogens (Fussi 2020). However, it is important to emphasize that pathogen control and management is a long-term task and an ongoing process based on research and collaboration among different actors and stakeholders. By implementing all of these measures, we will assist in the preservation of ash trees in the future by allowing natural adaptation processes, whether through natural regeneration or selection. Healthy ash populations that are able to adapt have the best chance of surviving extended periods of drought, pathogen pressure, or pest invasion.

9 Candidates individual contribution

- 1) **Eisen** A-K, Fussi B, Šikoparija B, Jochner-Oette S (2022) Aerobiological Pollen Deposition and Transport of *Fraxinus excelsior* L. at a Small Spatial Scale. *Forests* 13:424. <https://doi.org/10.3390/f13030424>

Barbara Fussi, Branko Šikoparija and Susanne Jochner-Oette contributed with suggestions for the conceptual development of the research question and the measurement network, corrections and proofreading. In addition, I received support in the preparation and counting of the slides from student assistants employed within the framework of the project. The majority of the work (**90 %**), from the construction of the field network and the installation of the gravimetric pole traps, to the data collection and statistical analysis, to the writing of the manuscript, was done by myself.

- 2) **Eisen** A-K, Semizer-Cuming D, Jochner-Oette S, Fussi B (2023) Pollination success of *Fraxinus excelsior* L. in the context of ash dieback. *Annals of Forest Science* 80:22. <https://doi.org/10.1186/s13595-023-01189-5>

The principal coordinators of the QuoVAPo project, Barbara Fussi and Susanne Jochner-Oette, had the idea for this study and developed the concept. DNA extraction and microsatellite marker genotyping were performed in the AWG laboratories. Devrim Semizer-Cuming provided the raw data for the advanced statistical analyses. Most of the work (**80 %**), from data collection in the study plots (sampling; recording of coordinates; vital assessment), statistical analyses using R, GIS analyses, and writing the manuscript, was done by me. All co-authors were involved in the final revision with corrections and proofreading.

- 3) **Eisen** A-K, Buchner L, Fussi B, Jochner-Oette S (2024) Does ash dieback affect the reproductive ecology of *Fraxinus excelsior* L.?. *Journal of Forestry Research* 35:16. <https://doi.org/10.1007/s11676-023-01670-x>

The idea for this study came from Susanne Jochner-Oette, supported by Barbara Fussi, PI of the AWG. Lisa Buchner helped with fieldwork and lab work on several days. All co-authors contributed with corrections and proofreading. I did most of the work (**90 %**), from data collection (field + lab work), to statistical analyses, to writing the manuscript myself.

- 4) Buchner L, **Eisen** A-K, Šikoparija B, Jochner-Oette S (2022) Pollen Viability of *Fraxinus excelsior* in Storage Experiments and Investigations on the Potential Effect of Long-Range Transport. *Forests* 13:600. <https://doi.org/10.3390/f13040600>

This publication was produced as part of Lisa Buchner's master's thesis, and she wrote the manuscript. Susanne Jochner-Oette and I supervised her and contributed with discussions, suggestions, corrections, and proofreading. The idea for the study came from Susanne Jochner-Oette, and some experimental parts were added by Branko Šikoparija. I helped with the methodological conceptualization. Lisa Buchner and I helped each other with the fieldwork. Overall, I did **25 %** of the work.

10 Publication list

Reviewed publications

1. **Eisen A-K**, Buchner L, Fussi B, Jochner-Oette S (2024): Does ash dieback affect the reproductive ecology of *Fraxinus excelsior* L.?. Journal of Forestry Research 35:16.
2. **Eisen A-K**, Semizer-Cuming D., Jochner-Oette S (2023): Pollination success of *Fraxinus excelsior* L. in the context of ash dieback. Annals of Forest Science 80:22.
3. **Eisen A-K**, Fussi B, Šikoparija B, Jochner-Oette S (2022): Aerobiological pollen deposition and transport of *Fraxinus excelsior* L. at a small spatial scale. Forests 13:424.
4. Buchner L, **Eisen A-K**, Šikoparija B, Jochner-Oette S (2022): Pollen viability of *Fraxinus excelsior* in storage experiments and investigations on the potential effect of long-range transport. Forests 13:600.
5. **Eisen A-K**, Fussi B, Jochner-Oette S (2022): Die Zukunft der Esche im Auwald. Auenmagazin 21: 4–9. ISSN 2190-7234.
6. Jochner-Oette S, Rohrer T, **Eisen A-K**, Tönnies S, Stammel B (2021): Influence of forest stand structure and competing understory vegetation on ash regeneration – potential effects of ash dieback. Forests 12:128.
7. **Eisen A-K**, Bussa M, Röder H (2020): A review of environmental assessments of biobased against petrochemical adhesives. Journal of Cleaner Production 277: 124277.
8. Bussa M, **Eisen A-K**, Zollfrank C, Röder H (2019): Life cycle assessment of microalgae products: State of the art and their potential for the production of polylactid acid. Journal of Cleaner Production 213: 1299-1312.

Conference Proceedings and Abstracts

Oral presentations

9. Buchner L, **Eisen A-K**, Köbölkuti Z, Böhm JW, Köpke K, Al Kubrusli R, Landgraf M, von Barga S, Fussi B, Kube M, Büttner C, Jochner-Oette S (2022): Detection of the influence of abiotic and biotic stressors on common ash using multisensorial and multitemporal data. IUFRO. All-Division 7 2022 Conference, 6th – 9th September in Lisabon, Portugal.
10. Böhm JW, **Eisen A-K**, Buchner L, Köbölkuti Z, Landgraf M, Köpke K, Al Kubrusli R, von Barga S, Fussi B, Jochner-Oette S, Büttner C, Kube, M (2022): Molecular monitoring of *Hymenoscyphus fraxineus*. IUFRO. All-Division 7 2022 Conference, 6th – 9th September in Lisabon, Portugal.

11. **Eisen A-K**, Fussi B, Šikoparija B, Jochner-Oette S (2021): Quo vadis Pollen? Untersuchungen zur Pollenausbreitung und Pollen- und Samenqualität bei der Gemeinen Esche hinsichtlich der Auswirkungen des Eschensterbens. FowiTA. Forstwissenschaftliche Tagung, 13th – 15th September 2021 in Freising, Deutschland.
12. Kahlenberg G, **Eisen A-K**, Jetschni J, Jochner-Oette, S (2021): Eignet sich die Blattphänologie der Esche als Frühindikator für eine Anfälligkeit gegenüber dem Eschtriebsterben?. FowiTA. Forstwissenschaftliche Tagung, 13th – 15th September 2021 in Freising, Deutschland.
13. **Eisen A-K**, Fussi B, Jochner-Oette S (2020): Investigations on pollen deposition and transport of *Fraxinus excelsior* L. at a small spatial scale. EAS. 7th European Symposium on Aerobiology, 16th – 20th November in Córdoba, Spain. Virtual Edition.

Poster presentations with Poster-Talk

14. **Eisen A-K**, Zollfrank C, Röder H (2018): Sustainability assessment of isocyanates as building blocks for polyurethanes using renewable starting materials: An LCA approach at the early stage of product development. SETAC Europe. 24th LCA Symposium, 24th – 26th September 2018 in Vienna, Austria.
15. **Eisen A-K**, Zollfrank C, Röder H (2018): Sustainability assessment of isocyanates as building blocks for polyurethanes using renewable starting materials: An LCA approach at the early stage of product development. 14. Ökobilanzwerkstatt - Aktuelle Trends in der Umwelt- und Nachhaltigkeitsbewertung, 10th – 11th Oktober 2018 in Osnabrück, Germany.

Poster presentations without Poster-Talk

16. **Eisen A-K**, Zollfrank C, Röder H (2018): Nachhaltigkeitsbewertung von Isocyanaten als Bausteine für Polyurethane mit erneuerbaren Ausgangsstoffen als Alternative zu erdölbasierten Materialien. Kooperationsforum Biopolymere, 24th October 2018 in Straubing, Germany.
17. **Eisen A-K**, Cyffla B, Kügel B (2016): Ökohydrologische Untersuchungen an ausgewählten Altarmen im Bereich der Stadt Eichstätt. 48. Jahrestreffen des Arbeitskreises Hydrologie, 17th -19th November 2016 in Munich, Germany.
18. **Eisen A-K**, Kügel B, Cyffka B (2013): Trophiebewertung und Nährstoffhaushalt des Kratzmühlsees, Landkreis Eichstätt, im Jahresverlauf bei Stagnation und Vollzirkulation. 45. Jahrestreffen des Arbeitskreises Hydrologie, 21st - 23rd November in Augsburg, Germany.

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