



# 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  $471.2 \pm 647.9$  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 (SO<sub>2</sub>), and nitrogen dioxide (NO<sub>2</sub>) reduce pollen viability. Increased ozone (O<sub>3</sub>) concentrations also have a negative effect on tree reproduction and regeneration (Darbah et al. 2008). In contrast, rising carbon dioxide (CO<sub>2</sub>) 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; Pliūra 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

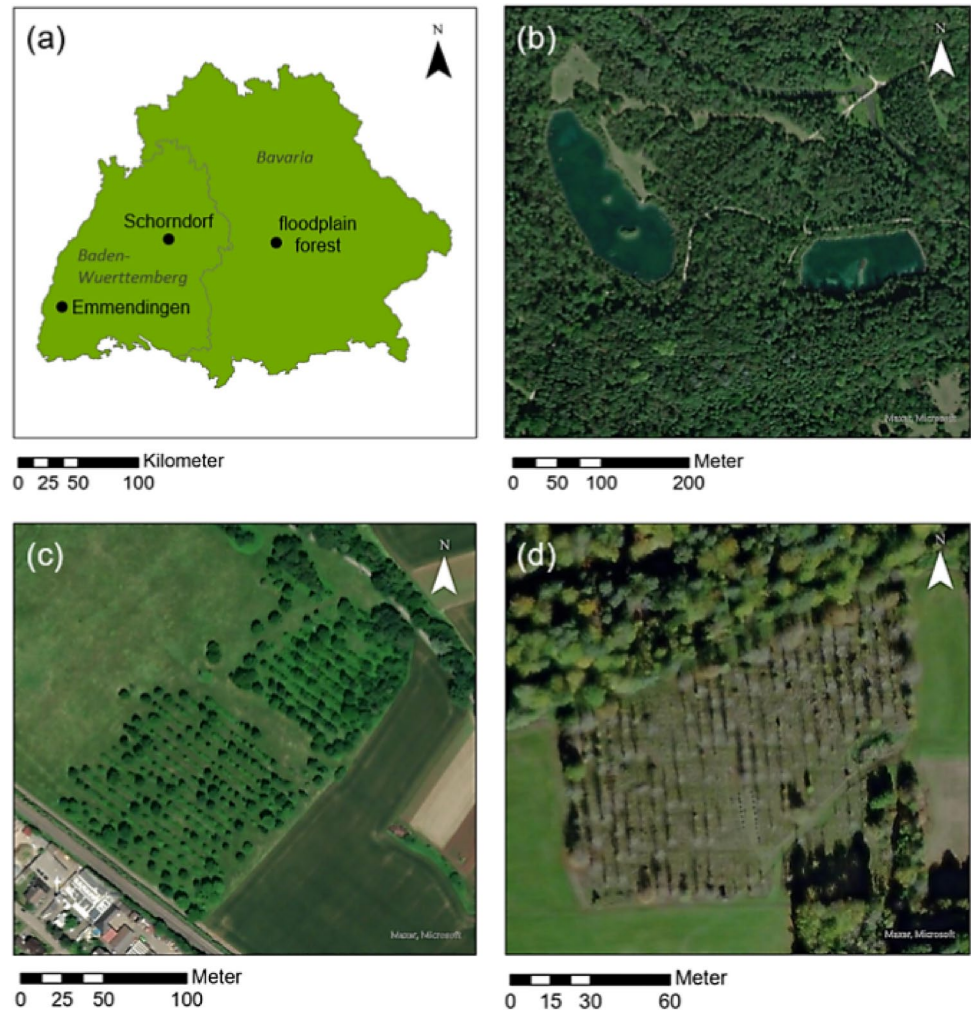
and stem quality before the outbreak of ash dieback (FVA 2006; Enderle et al. 2015). The floodplain forest is a natural landscape in which common ash has a share of approximately 15% (Jochner-Oette et al. 2021; Eisen et al. 2023).

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	Emmendingen 2020	Emmendingen 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  $\text{m}^3$  of the crown ( $50\text{ cm} \times 50\text{ cm} \times 50\text{ cm}$ ;  $0.125\text{ m}^3$ ) 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  $10\text{ }\mu\text{L}$  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 ( $P_{\text{inf}}$ ); (2) anthers level ( $P_{\text{fl}}$ ), (3) volume per  $\text{m}^3$  of crown ( $P_{\text{vol}}$ ) and (4) individual tree ( $P_{\text{in}}$ ) 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  $V_{\text{su}}$  is the volume of the suspension (25 mL),  $V_{\text{sa}}$  the volume of the sample,  $\bar{p}$  the average number of pollen grains per  $10\text{ }\mu\text{L}$  of suspension, fl the average number of anthers per inflorescence per tree,  $C_{\text{su}}$  the average number of inflorescences per  $\text{m}^3$  volume unit of the crown,  $M$  is the volume of the sampling unit ( $0.125\text{ m}^3$ ) 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  $4.6 \pm 2.3$  million pollen grains per  $P_{inf}$ ,  $10.8 \pm 5.1$  thousand pollen grains per  $P_{fl}$ ,  $2.0 \pm 1.6$  billion pollen grains per  $P_{vol}$ , and  $471.2 \pm 647.9$  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.

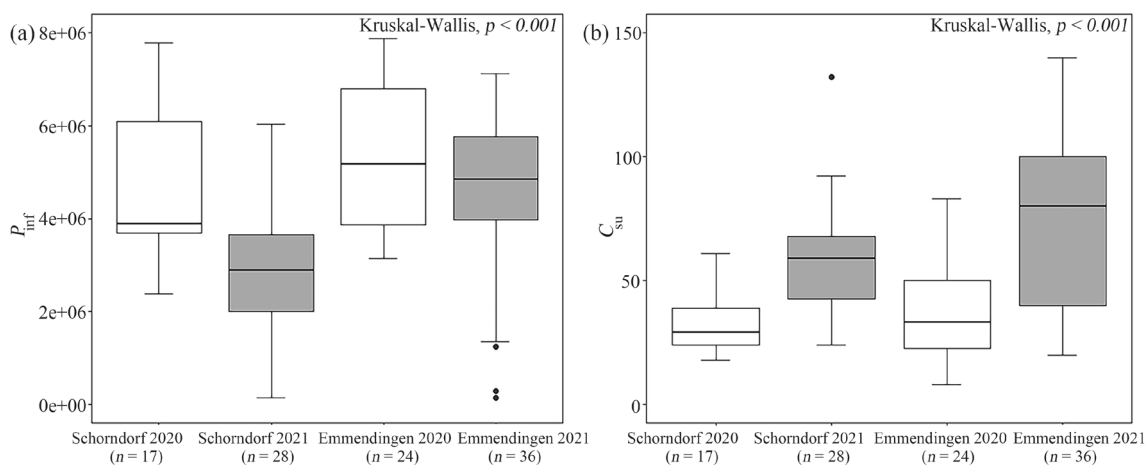
Average pollen production per inflorescence ( $P_{inf}$ ) for 2020 was  $5.2 \pm 2.0$  million pollen grains in Schorndorf and  $5.8 \pm 2.3$  million pollen grains in Emmendingen (Fig. 2 a). For 2021, the average  $P_{inf}$  in Schorndorf was  $2.0 \pm 1.2$

million pollen grains, in Emmendingen  $5.0 \pm 2.2$  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 ( $C_{su}$ ;  $0.125 \text{ m}^3$ ) 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  $33 \pm 12$  inflorescences per  $\text{m}^3$  in Schorndorf and  $35 \pm 18$  in Emmendingen. For 2021, an average  $C_{su}$  of  $59 \pm 22$  inflorescences per  $\text{m}^3$  was registered in Schorndorf and  $73 \pm 34$  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.

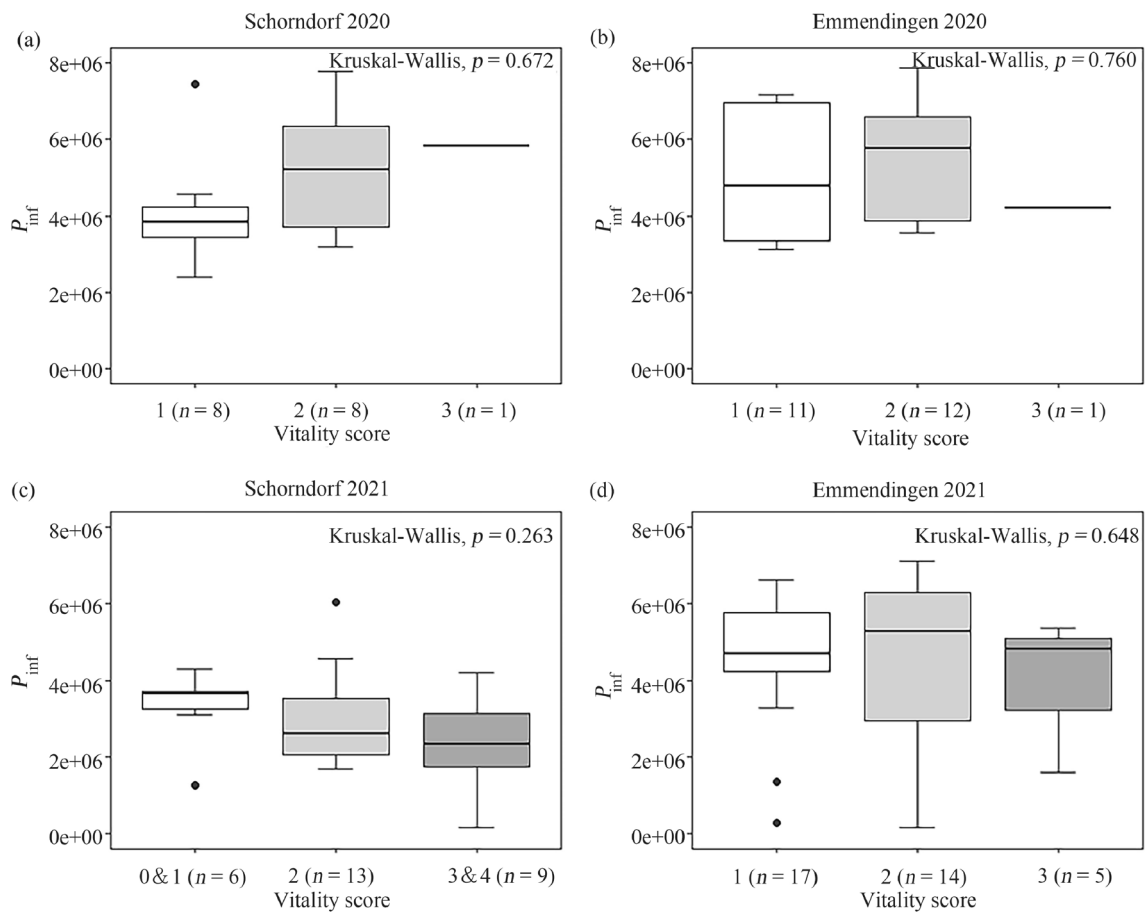
Pollen production per inflorescence ( $P_{inf}$ ) of healthy and diseased trees was not significantly different ( $p > 0.05$ ) (Fig. 3). For  $P_{fl}$ ,  $P_{vol}$  and  $P_{in}$ , there was no statistically significant difference (Appendix Table S1/Fig. S1–S3). However, for  $P_{inf}$  and  $P_{fl}$ , 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  $0.125 \text{ m}^3$  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;

( $p > 0.05$ ). 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

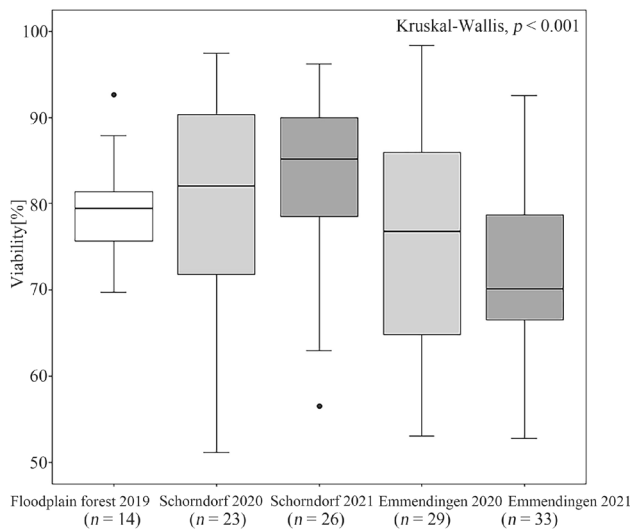
Pollen viability averaged  $73 \pm 17\%$  for viable,  $17 \pm 14\%$  for semi-viable and  $10\% \pm 8\%$  for non-viable regardless of year and location. In 2019, the average viability in the floodplain forest was  $79 \pm 6\%$ ; in 2020,  $76 \pm 19\%$  in Schorndorf and  $73 \pm 14\%$  in Emmendingen 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

$n$  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

(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–e). 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





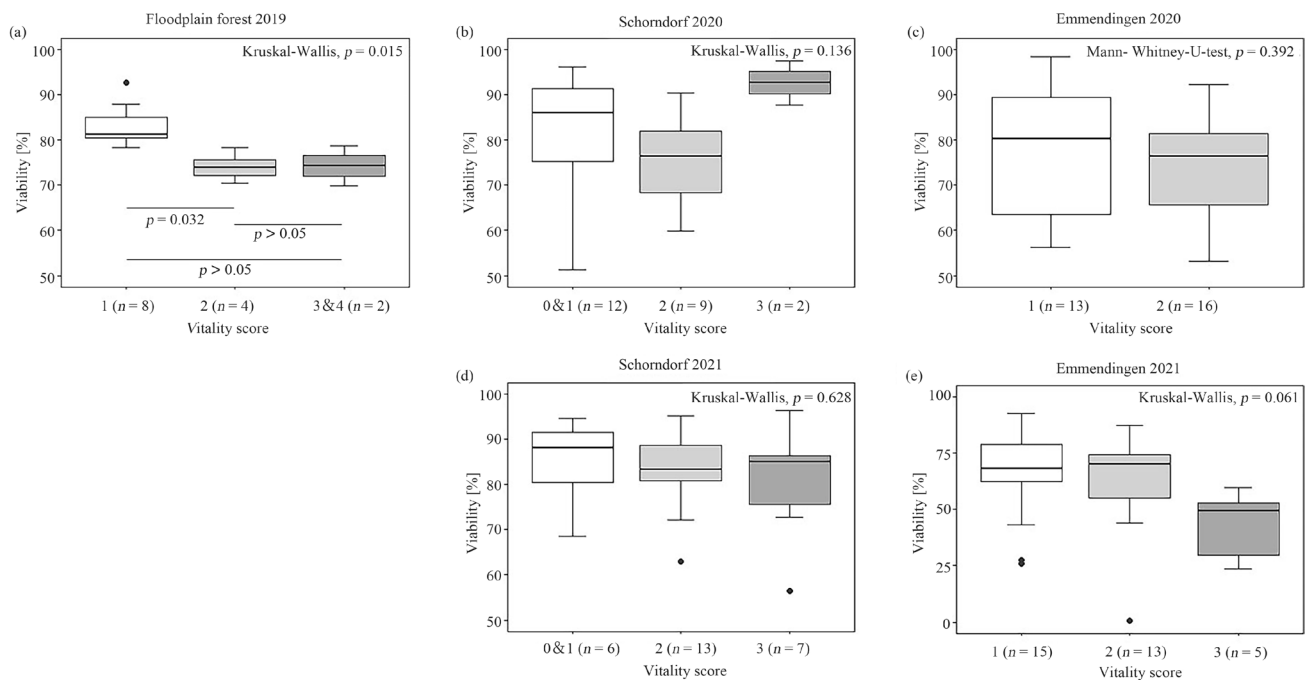
**Fig. 4** Boxplots of ash pollen viability for the floodplain forest, and for Schorndorf, and Emmendingen in 2019, 2020, and 2021; *n* indicates the number of trees sampled in each site. Interquartile range is represented by the height of the boxes, maximum and minimum values by the upper and lower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

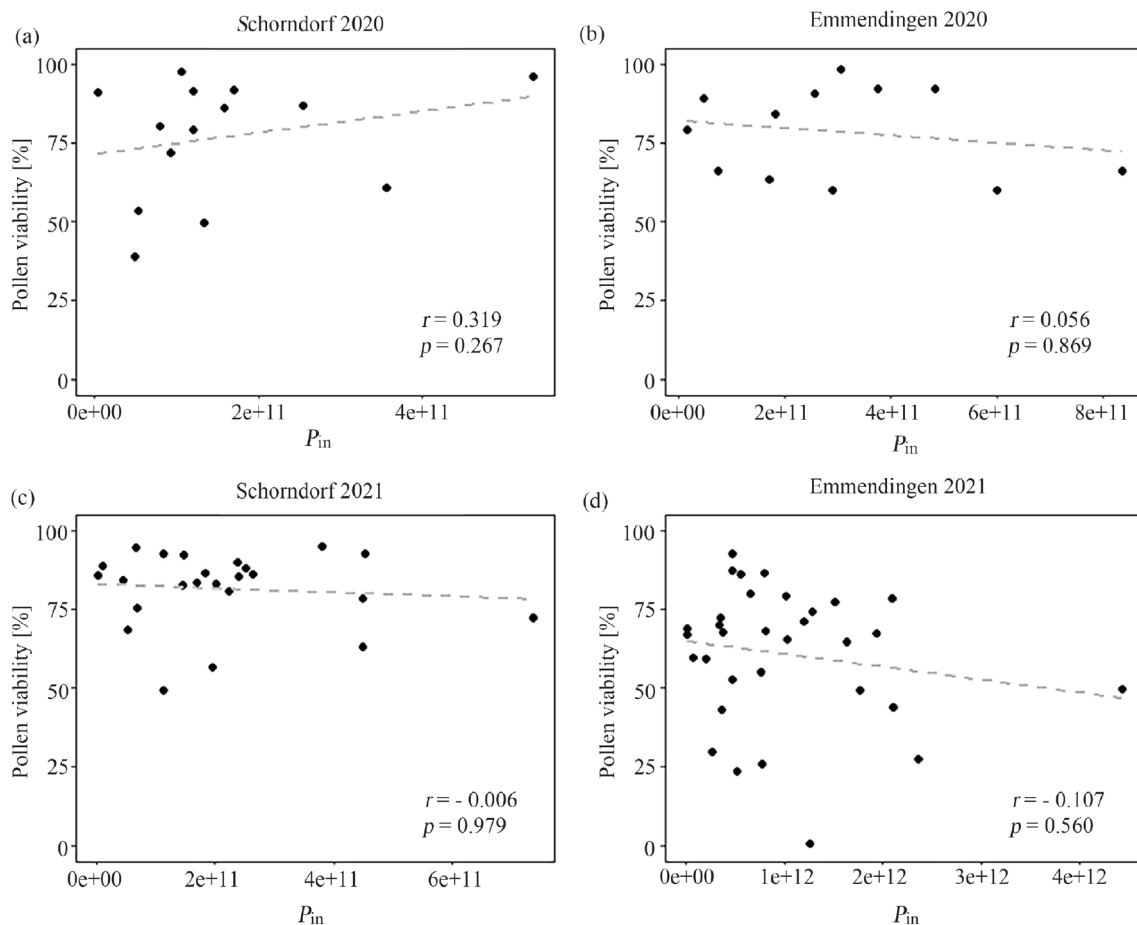
**Seed quality**

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

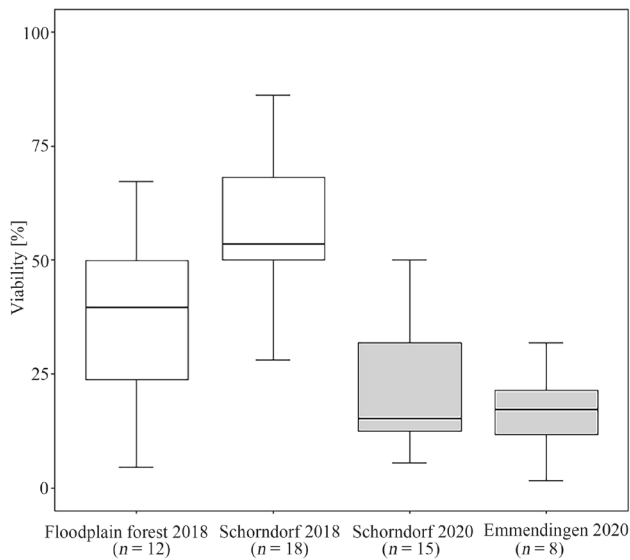
seeds from mother trees with vitality values of 0, 1, and 2 were harvested in Schorndorf in 2018. Generally, seed quality was not statistically different between healthy and diseased trees ( $p > 0.05$ ) (Fig. 8).

## 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

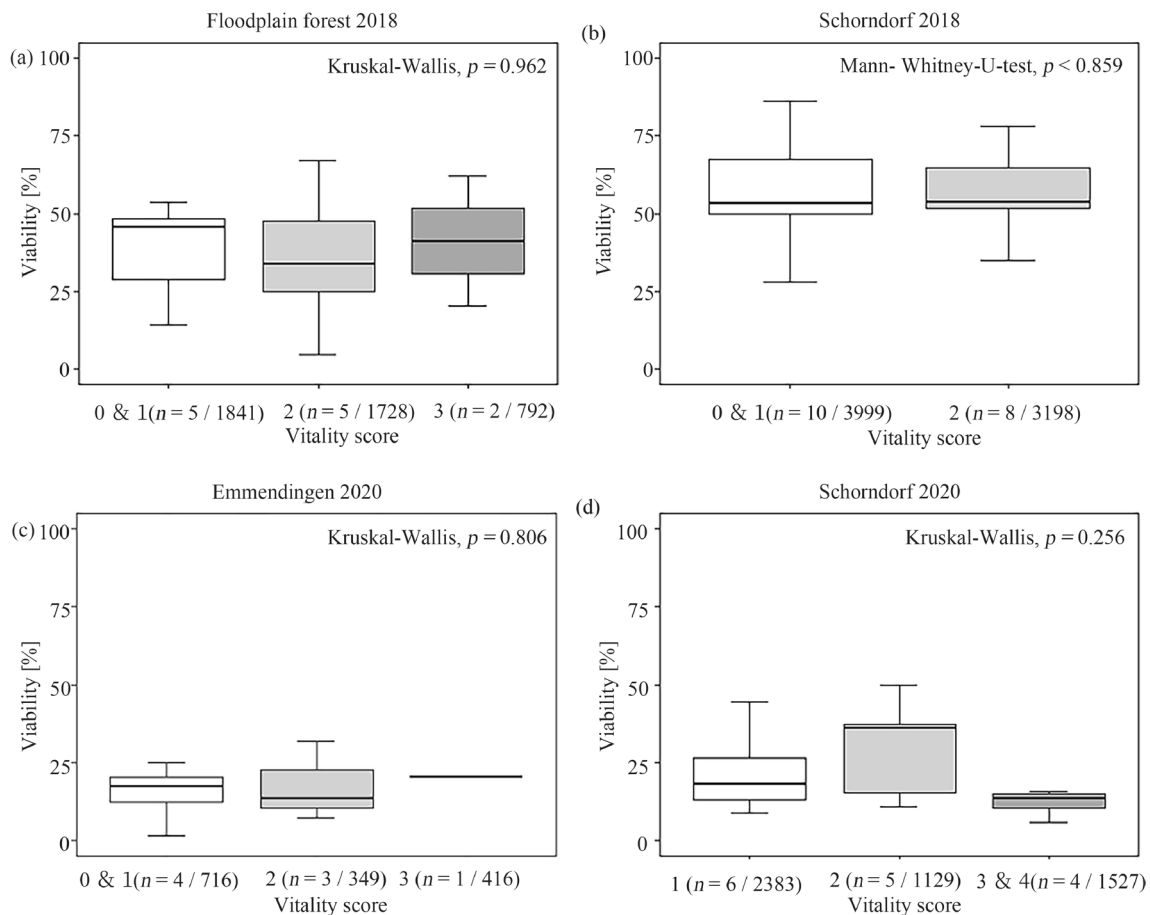
Environmental changes such as rising temperatures or increasing atmospheric carbon dioxide caused by climate change may influence pollen production and increase the reproductive potential (Bunce 2005; LaDeau and Clark 2006; D'Amato et al. 2007; Darbah et al. 2008; Zhang and Steiner 2022). Our estimated pollen production per anther was substantially low overall (10.8 cf. 27.7 thousand pollen grains) than that reported by Castiñeiras 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  $\text{NO}_x$  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 Castiñeiras 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 (Roloff 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).



## Outlook and recommendations for genetic conservation

Since our results have indicated that severely affected ash trees are unlikely to produce a high number of inflorescences, the diseased trees will not spread their genes to any significant extent to the next generation. Therefore, the negative effects on the ash gene pool due to dieback are expected to decrease in the future, given that enough healthy or less susceptible trees with high enough genetic variation remain and reproduce. It is more likely that future generations of natural regeneration that withstand inter- or intraspecific competition will be disease-resistant and provide opportunities for disease control as a product of natural selection (Metzler et al. 2012; McKinney et al. 2014; Jochner-Oette et al. 2021). Due to disease resistance can be partially inherited from parents, high mortality of ash trees combined with genetic variation in susceptibility could eventually lead to more resistant offspring (Kjær et al. 2012; McKinney et al. 2014; Lobo et al. 2015). Therefore, during thinning operations in ash stands, healthy and mildly damaged trees should be retained to maintain genetic diversity and thereby create the basis for future resistant populations. At the same time, targeted planting of ash identified as less susceptible could enrich the gene pool of existing stands and improve the resilience of populations (McKinney et al. 2014; Enderle et al. 2015). For this, we recommend the collection of scion materials for grafting and seeds from healthy trees and the establishment of seed orchards and genetic reserves for conservation of the species. Conservation through seed collection can create new and wide genetic variability since seeds are the product of sexual reproduction. Despite the increasing fragmentation of ash stands, these measures could contribute to the conservation of a wide range of genetic traits and ensure the long-term viability of ash. Maintaining genetic diversity not only ensures that the species survives, but also improves its ability to adapt and evolve to changing environmental conditions. Private forest owners can also aid in preserving and promoting natural resistance by planting seedlings from these seeds and maintaining dominant regeneration. However, it is important to emphasize that pathogen control and management is a long-term process based on research and collaboration among different stakeholders.

## Conclusions

A variety of factors, including the effects of ash dieback, influence the reproductive ecology of *F. excelsior*. The results show that crown damage caused by ash dieback does not lead to stress-induced increases in pollen production in the long term. However, diseased trees produced less flowers than healthy trees. In addition, there were no significant differences in pollen production and seed quality. However, the fact that

severely damaged trees showed a decrease in the intensity of flowering and pollen viability indicated a negative relationship between ash health and reproductive performance. Therefore, it is recommended promoting less susceptible ash trees and safeguarding their genetic material to ensure the long-term viability of ash populations. However, with at least 10% of the world's trees deemed susceptible to disease, biotic risk research and management, particularly in times of climate change (Goberville et al. 2016), remains highly relevant to ensure sustainable forest restoration in the future.

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