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# Does the Health Condition of the Common Ash Tree Affect Pollen Viability?

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**Abstract**

Pollen viability is a crucial determinant of reproductive success in plants. Given the enormous threat posed to the common ash (*Fraxinus excelsior* L.) by ash dieback, it is important to investigate the potential disease's effects on pollen viability and germination. Thus, we conducted an analysis of these pollen characteristics across three distinct forest stands in southern Bavaria, with up to 23 ash trees per study site. These ash trees exhibited varying degrees of ash dieback-related damage symptoms, enabling us to assess differences between mildly and severely affected trees (via Mann–Whitney-U/Wilcoxon tests, complemented by linear mixed-effects modelling). Pollen viability was assessed using the TTC test, while pollen germination capacity was evaluated on a sucrose–agar medium. Our findings revealed no statistically significant differences in pollen viability between mildly affected and severely diseased trees, as indicated by both the TTC test and pollen germination assay when applying non-parametric analyses (Mann–Whitney U and Kruskal–Wallis tests). Nevertheless, a consistent tendency towards higher pollen viability was observed in healthier ash trees. When accounting for the hierarchical structure of the data using linear mixed-effects modes, tree vitality showed a significant effect on pollen viability, whereas a substantial proportion of the observed variation was explained by interannual differences. These results indicate that ash trees generally retain the capacity to produce viable pollen across different levels of disease severity, but vitality-related effects are subtle and context-dependent. However, severely diseased trees produced few or no flowers, substantially reducing the likelihood that their pollen contributes to fertilization. We therefore conclude that ash dieback primarily limits reproductive success in common ash mainly by reducing flower and pollen production, whereas pollen viability itself is strongly driven by interannual differences. Consequently, no consistent pattern of declining pollen viability with increasing disease severity emerged.

**Keywords:** *Fraxinus excelsior* L.; ash dieback; forest pathology; pollen germination; TTC test; reproductive ecology; *Hymenoscyphus fraxineus*



Academic Editor: Rimvydas Vasaitis

Received: 5 May 2026
Revised: 12 June 2026
Accepted: 17 June 2026
Published: 19 June 2026



## 1. Introduction

Pollen play a crucial role in the reproductive cycle of forest trees, acting as a key vector for gene flow and genetic transfer to the next generation, thereby maintaining population vitality and diversity [1–5]. Most forest tree species are wind-pollinated (anemophilous) and thus produce large quantities of pollen and flowers to maximize fertilization chances [6–8]. Investigating pollen production provides insight into masting patterns, environmental

stress effects (e.g., air pollution, climate change), and tree health [9–12]. While pollutants such as $SO_2$, $NO_2$, and $O_3$ often have adverse effects on pollen production, $CO_2$ concentrations are often linked to advantageous effects [2,13]. Moreover, tree vigour is closely associated with the ability to produce flowers [12,14].

However, pollen quantity alone does not determine reproductive success; pollen viability—defined as "having the capacity to live, grow, germinate or develop"—is equally essential [15,16]. Due to the stochastic nature of pollen dispersal, viability and competitive interactions among pollen grains influence fertilization success and progeny quality [17–20].

Questions regarding reproductive processes are particularly relevant for *Fraxinus excelsior* L. (common ash tree), as this species is acutely threatened by ash dieback, a disease caused by the fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya [21], and is becoming increasingly fragmented in its populations [22–24]. The pathogen initially infects leaves and petioles before progressing to shoots and woody tissue, often resulting in tree death due to disrupted nutrient and water transport systems [24–29]. Recent studies indicated that approx. 10%–20% of the ash trees show reduced susceptibility [30,31], whereas previous studies reported proportions of only 1%–5% [23,32]. In addition, ongoing ash dieback may limit gene flow and pollination success and thereby substantially affect pollen viability and thus the reproductive ecology of *Fraxinus excelsior* L. [12,33–35]. A study by Bazhina and Aminev [36], which investigated the effect of *Biatorella difformis* on Scots pine, demonstrated that a pathogen can also have a significant impact on pollen germination and tube growth. They discovered that the fungus has the capacity to reduce pollen germination in infected trees by up to 25.2% and shorten tube length by up to 27.9%.

Natural regeneration is essential for population recovery, as resistance to ash dieback is genetically inherited and not population-specific [23,37–39]. Numerous studies have demonstrated substantial individual-level genetic variation in ash susceptibility [23,39–41], with heritability estimated of approx. 50% [23,42]. This implies that successful seed production is critical for regeneration, as seeds represent the outcome of fertilization combining genetic information from both parent trees via gamete fusion [20,43]. Although ash trees are functionally hermaphroditic, self-fertilized seeds do not usually develop, suggesting sub-dioecy associated with inbreeding depression [44]. Under favourable conditions, ash can produce large quantities of seeds [45,46], though germination rates remain moderate (58%–65%) [47,48]. Epigenetic mechanisms may also contribute to resistance development [49], making effective gene flow among resistant individuals a key factor for future regeneration [33,38].

A few prior studies already focused on ash pollen characteristics: Castiñeiras et al. [50] investigated pollen production and viability of *Fraxinus* in Spain, independent of ash dieback, while Buchner et al. [34] evaluated different methods for assessing pollen viability. Buchner et al. [34] further examined the influence of temperature and UV radiation on pollen viability, e.g., during long-distance transport, demonstrating an accelerated decline in viability under prolonged UV exposure and elevated temperatures. This methodological framework was subsequently applied in a field study by Eisen et al. [12]. The authors investigated whether ash dieback affects pollen production as well as pollen and seed viability in two seed orchards and a riparian forest. Regarding pollen viability, a slight tendency but no significant difference was detected between mildly affected and severely diseased ash trees in the two seed orchards. However, a significantly higher pollen viability was found for less affected ash trees in the riparian forest. Due to these disparate results, the present study aimed to conduct a more detailed investigation focusing on three different forest stands in Southern Germany, Bavaria, over two consecutive years. Specifically, we tested whether ash dieback and associated differences in tree health have an impact on pollen quality in different forest stands.

## 2. Materials and Methods

### 2.1. Study Site

Our investigations were conducted in three Bavarian forest stands (Figure 1a): BY1 Monheim (48°48′ N, 10°47′ E, 470 m a.s.l.; Figure 1b), BY2 Bruckberg (48°28′ N, 11°57′ E, 419 m a.s.l; Figure 1c), and BY3 Isen (48°11′ N, 12°5′ E, 519 m a.s.l.; Figure 1d). The sites BY1 and BY3 represented typical deciduous, broad-leaved mixed forests, whereas BY2 was a riparian forest. The average annual air temperature (1991–2020) was 8.9 °C at BY1 (German Weather Service (DWD) station Harburg) and 8.7 °C at both BY2 (DWD station Munich Airport) and BY3 (DWD station Ebersberg). The average annual precipitation sums for the same reference period were 824 mm (BY1), 757 mm (BY2) and 1005 mm (BY3), respectively. At each study site, 12 to 23 ash trees of varying health status were selected (Table 1). Trees were chosen based on accessibility using a lifting platform. The health status of the trees was classified according to a standardized vitality scoring system for ash trees affected by ash dieback [51]. Vitality class 0 represents healthy trees without any visible infection symptoms or disease-associated leaf loss. In class 1, initial symptoms such as reduced foliage are present, but dead twigs are absent. Class 2 is characterized by more pronounced leaf loss and the occurrence of the first dead twigs. In class 3 and 4, the proportion of dead wood increases progressively, and leaf biomass is substantially reduced. Class 5 represents dead trees. The vitality assessments for both 2021 and 2022 were conducted at the end of July, when ash trees typically have reached their maximum leaf mass [52]. Table 1 summarizes the numbers of trees investigated per site and year, along with their associated vitality classes. In 2022, samples were taken from the same trees as in 2021; however, the number of trees in each vitality class varied through the years and sites due to differences in tree health, and flower availability, which was higher in 2022. Germination analyses in 2022 were conducted using pollen from the same trees as for the TTC analysis. For statistical analyses, trees were grouped into mildly affected (vitality classes 1 and 2) and severely affected individuals (vitality classes 3 and 4). Vitality class 0 was excluded due to the absence of completely healthy trees across all sites. Trees assigned to vitality class 4 constituted the smallest sample group in both study years, primarily due to our observation that many individuals at this stage no longer produced flowers.

### 2.2. Pollen Viability

The TTC test (2,3,5-triphenyltetrazolium chloride) is commonly used to determine cellular metabolic activity, as triphenyltetrazolium chloride induces a colour change in living cells based on the presence of active dehydrogenase enzymes. Accordingly, the TTC test assesses the respiratory activity of tissues and is therefore suitable for evaluating the pollen viability [53]. The redox reaction results in the formation of red formazan, which does not occur in non-viable pollen grains. In this manner, viable pollen can be easily distinguished from non-viable pollen under a light microscope. Several studies have already confirmed that this approach is suitable for ash pollen [12,34,50]. The 1% TTC staining solution was prepared by dissolving 1 g of 2,3,5-triphenyltetrazolium chloride and 60 g sucrose in 100 mL of distilled water, following Shivanna and Rangaswamy [54].

A few days prior to the beginning of the ash pollen seasons in 2021 and 2022, we selected seven twigs with closed flowers that were cut from up to 23 ash trees per site and year (see Table 1). Twigs were sampled at different heights and from at least three cardinal orientations per tree. The cut twigs were placed in vases filled with 100 mL tap water and stored at room temperature in the laboratory. After 24 to 30 h, when anther dehiscence had begun, pollen was harvested by gently shaking the flowers and collecting the released pollen on microscope slides. For each tree, five slides were prepared using pollen pooled from all sampled twigs per tree. Two drops of the TTC solution were added

to each slide, which were subsequently sealed with cover slips and placed on moistened filter paper in Petri dishes (90 mm diameter) at room temperature in darkness. After 24 h of incubation, the viable pollen was quantified under a light microscope (Olympus CX23, Olympus Corperation, Tokio, Japan, magnification 40×). Viability was quantified as the percentage of red-stained (viable) pollen grains relative to a total of 400 pollen grains per slide.

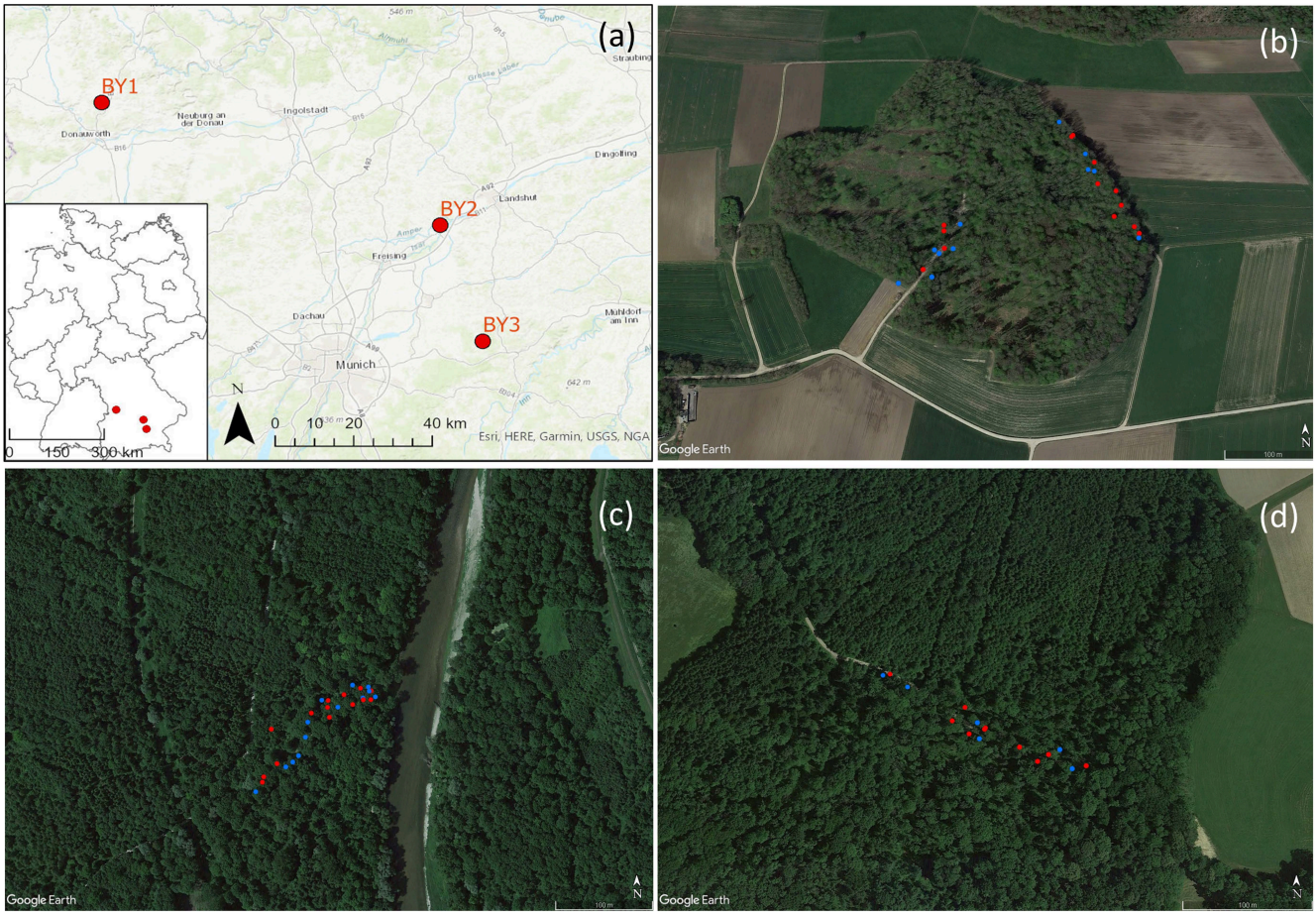


**Figure 1.** Study sites: (**a**) the location of the three study sites in Bavaria, Germany, (**b**) Monheim BY1, (**c**) Bruckberg BY2 and (**d**) Isen BY3. The dots indicate the investigated ash trees, blue dots represent mildly affected trees (vitality classes 1 and 2), and red dots represent severely affected trees (vitality classes 3 and 4).

**Table 1.** The number of ash trees per vitality class at the study sites BY1 (Monheim), BY2 (Bruckberg) and BY3 (Isen) in the years 2021 and 2022.

| | 2021 | | | | | 2022 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Class 1** | **Class 2** | **Class 3** | **Class 4** | **Sum** | **Class 1** | **Class 2** | **Class 3** | **Class 4** | **Sum** |
| BY1 | 4 | 11 | 4 | 2 | 21 | 3 | 8 | 8 | 2 | 21 |
| BY2 | 3 | 8 | 5 | 2 | 18 | 3 | 9 | 10 | 1 | 23 |
| BY3 | 4 | 2 | 5 | 1 | 12 | 1 | 6 | 9 | 1 | 17 |

### *2.3. Pollen Germination*

Pollen germination experiments were carried out exclusively in 2022 using the same trees and twigs as those sampled for pollen viability assessment. Pollen was collected from opened flowers into Petri dishes and stored in a freezer (−80 °C) until further analyses

were conducted in mid-May. We followed the approach outlined by Buchner et al. [34], which was based on a modified approach originally introduced by Fröhlich [55]. The nutrient medium was prepared by dissolving 10 g sucrose, 1 g agar, 0.002 g boric acid in 89 mL of distilled water, followed by heating to boiling. Subsequently, 14 mL of the molten medium was added to cover the bottom of a Petri dish (90 mm diameter). After cooling and solidification of the medium, a fine layer of pollen was sprinkled onto the surface using a brush. The Petri dishes were then stored in complete darkness at 25 °C and 80% relative humidity in a climate chamber (KBWF, Binder GmbH, Tuttlingen, Germany). After 24 h of incubation, the germinated pollen was quantified under the light microscope (Olympus CX23 Olympus Corperation, Tokio, Japan, magnification 40×). The number of germinated pollen grains was noted from a total of 400 counted pollen grains per Petri dish. Pollen grains were considered germinated when the pollen tube length equalled or exceeded the diameter of the pollen grain [56].

### *2.4. Statistical Analysis and Artificial Intelligence*

As the Shapiro–Wilk normality test indicates non-normal data distribution, the Mann–Whitney-U/Wilcoxon test was employed to calculate the differences between the two groups, mildly affected (vitality class 1 and 2) and severely affected (vitality class 3 and 4) trees. The Kruskal–Wallis test (>2 variables) followed by a post hoc test (Wilcoxon signed-rank test) determined if the differences between pollen quality and the years or locations were statistically significant. In addition, a Linear Mixed Model was applied to estimate the effect of tree vitality on pollen viability while accounting for random effects of tree identity, study site and year, thereby addressing hierarchical data structure. Spearman's rank correlation coefficient was utilized for correlation analyses. All statistical analyses were performed in R (RStudio Version 4.2.2). Boxplots were generated using the ggplot2 package [57]. A sensitivity analysis was conducted using the pwr package [58] to assess the detectable effect size given the available sample siz. During the preparation of the manuscript, DeepL Write (DeepL SE Version Next-Generation Language Model) and ChatGPT (OpenAI Version GPT-5.5) were used to improve the language and readability of certain passages. No AI tools were used for data analysis, data interpretation, or the generation of scientific content.

## 3. Results

### *3.1. Pollen Viability*

#### 3.1.1. General Patterns Across Sites and Years

In 2022, the average percentage of viable pollen (94.4%) was 18% higher than the average in 2021. In 2021, study site BY3 exhibited the highest number of viable pollen, with an average of 73.8%, surpassing BY2 by 1% and BY1 by 6%. Conversely, in 2022, BY2 showed the highest average of viable pollen with 94%, compared to BY1 (−5%) and BY3 (−10%). A Kruskal–Wallis test revealed a statistically significant effect of year on pollen viability across all study sites ($p < 0.001$; Figure 2). Post hoc comparisons indicated significant interannual differences within each study site (BY1: 2021 vs. 2022, $p < 0.001$; BY2: 2021 vs. 2022, $p < 0.001$; BY3: 2021 vs. 2022, $p = 0.003$). When comparing study sites within individual years, no statistically significant differences were observed in 2021. In contrast, in 2022 significant differences were detected between BY1 and BY2 ($p = 0.041$) and between BY3 and BY2 ($p = 0.005$).

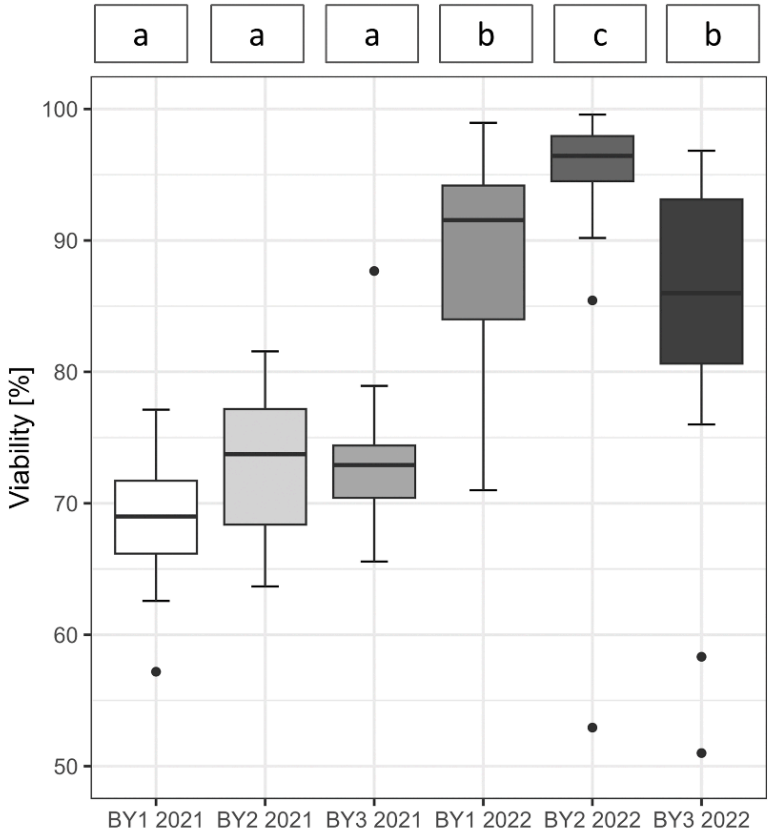


**Figure 2.** Boxplots of ash pollen viability per tree for the study sites BY1 (Monheim), BY2 (Bruckberg) and BY3 (Isen) in the year 2021 and 2022. Different shading indicates study sites and years. The interquartile range is represented by the box height, whiskers indicate minimum and maximum values, bold horizontal lines denote medians, and points indicate outliers. Different letters, a, b and c, indicate statistically significant differences between study sites within the same year (post hoc test, $p < 0.05$).

#### 3.1.2. Differences Between Vitality Classes

Comparisons between mildly affected (vitality classes 1 and 2) and severely affected trees (vitality class 3 and 4) indicated that, in most cases, less affected trees tended to exhibit higher pollen viability, especially in 2022 (Figure 3a). However, these differences were not statistically significant ($p > 0.05$) in any of the analyzed comparisons (2021: BY1 $p = 0.697$, BY2 $p = 0.820$, BY3 $p = 0.423$; 2022: BY1 $p = 0.324$, BY2 $p = 0.578$, BY3 $p = 0.417$; Mann–Whitney-U/Wilcoxon test). A more detailed comparison among individual classes (Figure 3b) revealed that trees in vitality class 1 tended to show the highest pollen viability. However, the Kruskal–Wallis test indicated no statistically significant differences among vitality classes. The results based on single vitality classes should be interpreted with caution due to small and uneven sample sizes within individual vitality classes across sites and years (see Table 1).

The results of the linear mixed-effects model (Table 2) indicated that tree vitality class had a significant effect on pollen viability after accounting for random effects of site, year, and individual tree. Using vitality class 1 as the reference category (mean pollen viability: 85.5%), trees in vitality class 2 exhibited on average 7.5% lower pollen viability, followed by vitality class 3 (−5.3%) and vitality class 4 (−6.8%). These effects were statistically significant for vitality classes 2 ($p = 0.002$) and classes 3 ($p = 0.034$), while the effect for vitality class 4 was marginally non-significant ($p = 0.051$). Variance partitioning revealed that interannual variability accounted for the largest proportion of total variance (171.03), whereas variance attributable to individual trees (6.55) and study sites (9.44) was

comparatively small. Overall, these results indicate that reduced tree vitality is associated with lower pollen viability, although this effect occurs against a background of strong interannual variability.

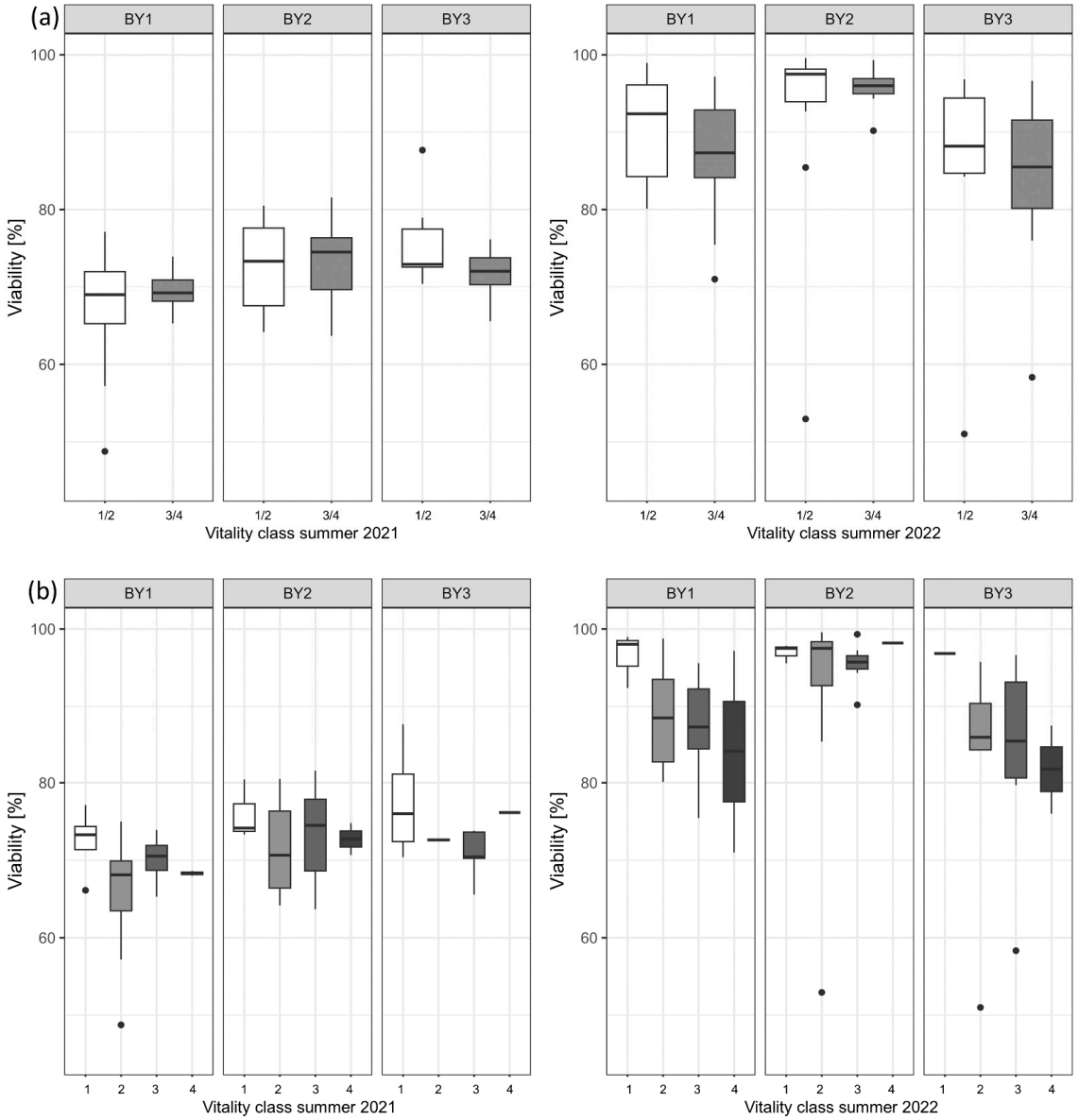


**Figure 3.** (**a**) Boxplots showing the percentage of viable pollen per tree for mildly affected (vitality classes 1 and 2) and severely affected trees (vitality classes 3 and 4) and (**b**) boxplots showing the percentage pollen viability across individual vitality classes (1–4) for study sites BY1 (Monheim), BY2 (Bruckberg) and BY3 (Isen) in the year 2021 and 2022. The interquartile range is represented by the box height, whiskers indicate minimum and maximum values, bold horizontal lines denote medians, and points indicate outliers.

**Table 2.** Results of the linear mixed-effects model assessing the effect of tree vitality class on pollen viability. Vitality class 1 was used as the reference category. Fixed-effect estimates represent differences in pollen viability (%) relative to the reference class. Variance components of the random effects are reported for year, site, and individual tree.

| Effect | Estimate (%) | SE | *p*-Value |
|---|---|---|---|
| **Fixed effects** | | | |
| Intercept (Vitality class 1) | 85.5 | 9.64 | — |

**Table 2.** *Cont.*

| Effect | Estimate (%) | SE | *p*-Value |
|---|---|---|---|
| Vitality class 2 | −7.5 | 2.40 | 0.002 |
| Vitality class 3 | −5.3 | 2.46 | 0.034 |
| Vitality class 4 | −6.8 | 3.44 | 0.051 |
| **Random effects** | **Variance** | | |
| Year | 171.03 | | |
| Site | 9.44 | | |
| Tree | 6.55 | | |

### *3.2. Pollen Germination*

#### 3.2.1. Differences Between Vitality Classes

As previously mentioned, pollen germination was assessed only in 2022. The proportion of germinated pollen was comparable between study sites BY2 (11.8%) and BY3 (11.4%), but substantially lower at BY1 (6.7%). Overall, the germination pattern broadly mirrored that observed from pollen viability: pollen from mildly affected trees (vitality classes 1 and 2) tended to show higher germination rates (Figure 4a). However, this tendency was only marginally significant at study site BY2 ($p$ = 0.079; Mann–Whitney-U test) and not statistically significant at BY1 ($p$ = 0.231) and BY3 ($p$ = 0.688). A more detailed comparison among individual vitality classes did not reveal a consistent pattern across study sites (Figure 4b). Although trees in vitality class 1 at BY1 and BY3 tended to exhibit higher germination rates compared to more severely affected trees, the Kruskal–Wallis test confirmed no statistically significant differences among the four vitality classes.

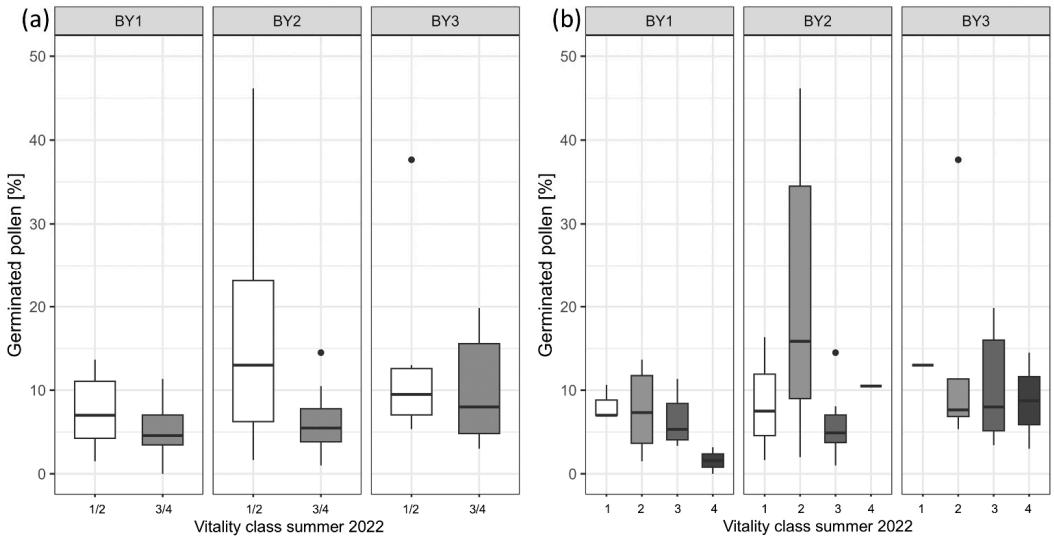


**Figure 4.** Boxplots showing the percentage of germinated pollen for the two groups related to a different health status (vitality class 1/2: mildly affected; 3/4: severely affected) (**a**) and the percentage of the germinated pollen for all groups related to the vitality class 1 to 4 (**b**) for the study sites BY1 (Monheim), BY2 (Bruckberg) and BY3 (Isen) in the year 2022. 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, and points indicating outliers.

To increase statistical power given limited and uneven sample sizes within individual vitality classes, data from all three study sites were pooled for additional exploratory analyses (Figure 5). This approach is supported by results from the linear mixed-effects model, which showed that interannual variability accounted for the largest proportion of total variance in pollen viability, whereas variance attributable to study sites was compara-

tively small. The pooled comparison between mildly (vitality classes 1 and 2) and severely affected (vitality class 3 and 4) trees indicated no statistically significant difference in pollen viability for either year (2021: $p = 0.800$; 2022: $p = 0.194$; Mann–Whitney-U/Wilcoxon test, Figure 5a). For pollen germination in 2022, a marginal difference was observed ($p = 0.069$), indicating a tendency towards higher germination rates in mildly affected trees. When comparing all four vitality classes using the Kruskal–Wallis test, marginal differences in pollen viability were detected for both years (2021: $p = 0.062$; 2022: $p = 0.088$; Figure 5b). Post hoc analyses suggested that these patterns were primarily driven by differences between vitality classes 1 and 2 in 2021 ($p = 0.053$) and between classes 1 and 3 in 2022 ($p = 0.016$). However, given the marginal significance levels and the exploratory nature of this pooled analysis, these results should be interpreted with caution. Although statistical power may be limited for comparisons involving the smallest group, the consistently high $p$-values across all pairwise Wilcoxon tests ($p \geq 0.820$, for all areas and both years) and the extremely small overall effect size ($\eta^2[H] = 0.003$) suggest that there is no meaningful difference in pollen viability among vitality classes. Based on the smallest group (n = 9; vitality class 4), the minimum detectable effect size was estimated for a two-sample comparison with 80% power and $\alpha = 0.05$. The analysis indicated that only very large effects (Cohen's d $\approx 1.41$) could be detected.

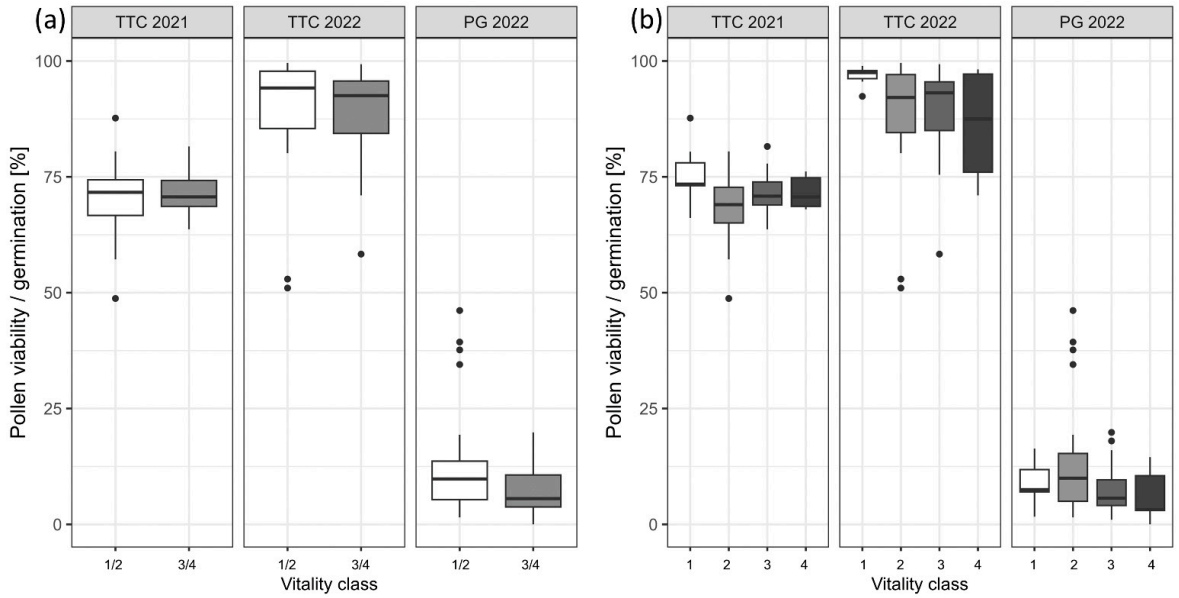


**Figure 5.** Boxplots showing the percentage of the pollen viability for the years 2021 and 2022 (TTC 2021/TTC 2022) and the percentage of germinated pollen (PG 2022) for the two groups related to a different health status (vitality class 1/2: mildly affected; 3/4: severely affected) (**a**) and for all groups related to the vitality class 1 to 4 (**b**), based on pooled data from all study sites. 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, and points indicating outliers.

For pollen germination in 2022, no statistically significant differences among the four vitality classes were detected ($p = 0.268$).

#### 3.2.2. Correlations Analysis of Pollen Viability and Germination

Correlation analyses between pollen viability and pollen germination per tree in 2022 revealed a weak, non-significant negative relationship when pooling data from all study sites (Spearman's $\rho = -0.025$, $p = 0.848$; Figure 6). When analyzed separately by study site, BY1 and BY3 showed weak positive but non-significant correlations, whereas BY2

exhibited a weak negative, non-significant correlation (BY1: Spearman's ρ = 0.323, $p = 0.153$; BY2: Spearman's ρ = −0.178, $p = 0.417$; BY3: Spearman's ρ = 0.191, $p = 0.461$).

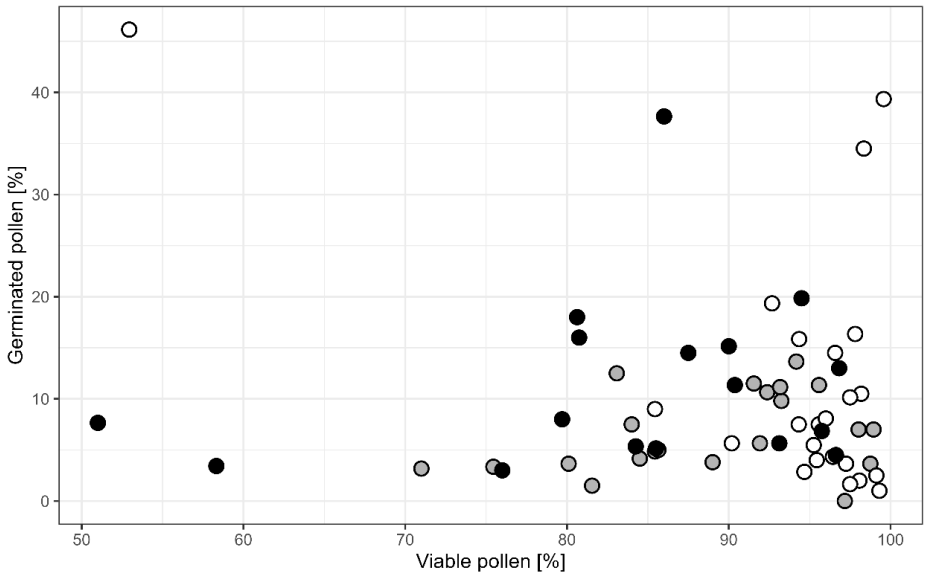


**Figure 6.** Scatterplots of the percentage of viable pollen [%] and the percentage of germinated pollen [%] for the study sites BY1 (grey), BY2 (white) and BY3 (black) in 2022.

## 4. Discussion

Pairwise comparisons based on TTC tests and pollen germination did not reveal statistically significant differences between mildly and severely affected ash trees. However, a consistent tendency towards higher pollen viability and germination in less affected trees was observed. This pattern became more evident when accounting for hierarchical data structure in a linear mixed-effects model, including year, site and tree identity as random effects, which identified a significant effect of tree vitality on pollen viability. In contrast, the non-parametric analyses did not detect significant differences among vitality classes. Overall, these findings indicate that pollen viability and germination were primarily driven by strong interannual variability, while effects of tree vitality were more subtle and only detectable within a model-based framework. The large variance attributed to year suggests that temporal variation may have masked health-related differences in the non-parametric analyses.

In prior studies, the effects of plant diseases on pollen viability have rarely been investigated to date. Kazimierski and Kazimierska [59] demonstrated that viral infections in *Lupinus luteus* L. resulted in significantly reduced rates of pollen germination and pollen tube growth, while healthy plants exhibited higher reproductive performance. Conversely, viral infection led to degeneration of components within the embryo sac at various developmental stages.

Eisen et al. [12] investigated the influence of ash dieback on the pollen viability of *Fraxinus excelsior* L. in two seed orchards and one riparian forest in southern Germany. Similar tendencies of reduced pollen viability in severely affected trees were observed, although statistically significant differences were detected only between mildly and severely diseased ash trees within the riparian forest. Taken together, these findings indicate that ash trees retain the capacity to produce viable pollen irrespective of their health status. Consequently, ash dieback disease does not appear to universally impair fertilization potential at the pollen level. Even pollen from severely affected trees and potentially carrying alleles associated with high susceptibility remains functionally capable of fertilization.

However, as ash dieback is known to promote the formation of epicormic shoots during disease progression. The potential influence of such shoots on pollen quality remains poorly understood and warrants targeted investigations. While pollen production per inflorescence appears largely unaffected by disease severity [12], flower production itself was markedly reduced in severely affected trees. This was confirmed in our study, where pollen could be collected from only nine out of 112 trees assigned to vitality class 4 across all study sites and years (see Section 2). As a result, the probability of pollen from severely diseased trees contributing to fertilization events is substantially reduced. These findings align with prior research using genetic analyses and mating models demonstrating that ash dieback is linked to a diminished individual reproductive success of ash trees [35,39].

The mean pollen viability of *Fraxinus excelsior* assessed in this study exceeded values reported by Eisen et al. [12] in southern Germany and by Castiñeiras et al. [50] in Spain (81.1% vs. 73% and 65%, respectively). This variation likely reflects local environmental conditions, including temperature and radiation regimes influencing pollen viability across the study sites in Germany and Spain [60,61]. Elevated temperatures and prolonged UV exposure are known to accelerate pollen viability loss [62,63], an effect that is particularly pronounced in Spain and in seed orchards, where pollen are likely exposed to higher temperatures and increased solar radiation compared to enclosed forest environments characterized by cooler and more buffered microclimatic conditions. Buchner et al. [34] demonstrated a rapid decline in ash pollen viability under elevated temperatures and UV radiation. High UV radiation has also been shown to adversely affect pollen viability in other plant species [64,65]. Consequently, prevailing meteorological conditions can exert a strong influence on pollen viability [66]. According to Ge et al. [66], the viability of *Panicum virgatum* L. pollen declined fivefold faster under sunny conditions than under overcast conditions. In the present study, twigs bearing male flowers, from which pollen were extracted, were stored in the laboratory under uniform light conditions without direct solar radiation exposure. Therefore, the influence of radiation on pollen viability is likely negligible. Accordingly, this experimental setup appears well suited to assess the influence of ash dieback, as confounding effects of solar radiation on pollen viability were largely minimized.

Air pollution, such as carbon monoxide (CO), sulphur dioxide ($SO_2$) and nitrogen dioxide ($NO_2$), has been linked to negative impacts on pollen viability [67]. Investigations into pollen viability conducted in both highly polluted regions characterized by heavy traffic and in unpolluted areas have demonstrated distinct differences, with notably diminished viability observed in more polluted areas [68–72].

However, these effects are species-specific, with some pollen types exhibiting greater tolerance to air pollution than others [53,67]. Although air pollution was not explicitly quantified in this study, its potential influence on pollen viability warrants consideration. The study sites Monheim (BY1) and Isen (BY3) are located at a considerable distance from major roads and are therefore less likely to be substantially affected by traffic related air pollution.

By contrast, the ash trees located in Bruckberg (BY2) are situated adjacent to an industrial area. Nevertheless, pollen viability at this site was comparatively high across both study years, particularly in 2022. This suggests that air pollution was not a dominant driver of pollen viability at this location. In order to draw further conclusions about the influence of pollution but also of other external factors, such as local site conditions, viruses or parasites, it would be beneficial to incorporate these factors into future research. Instead, pollen viability may be influenced by phenotypic differences among trees. Gupta et al. [73] found that in plant species with dichasial inflorescences (comprising three sequential flowering stages), functionally male and/or hermaphroditic flowers exhibit higher pollen viability. A

similar effect may apply to ash trees due to their trioecious nature. In this study, pollen was collected from trees with male flowers only; however, due to the absence of confirmatory testing, it cannot definitively be excluded that some sampled trees were hermaphroditic. Notably, higher proportions of male/hermaphroditic individuals compared to male ash trees were sampled at the BY2 site. Furthermore, the sexual characteristics of ash trees are known to vary from year to year [30,74], which may additionally contribute to interannual differences in pollen viability, as determined using the TTC test in 2021 and 2022. Moreover, the results indicate that pollen viability is not determined solely by tree vitality. The linear mixed-effects model revealed a strong effect of year, suggesting that environmental factors such as temperature [34], humidity [63], pollution [53,67] and nitrogen supply [15] contribute substantially to the observed differences between 2021 and 2022.

Methodologically, the TTC test serves as an indicator of potential pollen quality, whereas pollen germination reflects the actual capability of pollen grains to germinate under defined conditions and thus provides a more direct measure of potential reproductive performance [62]. Consequently, a direct quantitative comparison between the results of the TTC test (estimation) and pollen germination rates is not feasible. A substantially lower proportion of germinated pollen compared to TTC-derived viability is therefore expected and was also observed in this study (averaged across all sites in 2022 10.03% vs. 89.41%). The lower germination rates observed in our study may reflect species-specific germination requirements or limitations of the in vitro assay conditions, which are not captured by TTC staining. Similar discrepancies between staining-based viability estimates and germination success have been reported in previous studies [75–78]. Şensoy et al. [79] attributed the observation of higher viability rates using chemical methods, such as TTC, to the capacity of these methods to also stain immature pollen. Furthermore, Moore and Janick [80] assumed the dye method may overestimate viability, while in vitro tests may underestimate germination. Impe et al. [81] suggested that pollen germination may be partly under genetic control. Correspondingly, previous studies investigating the relationship between pollen viability and germination have yielded inconsistent results: Oberele and Watson [82] reported only weak correlations between these two parameters, while Parfitt and Ganeshan [75] as well as Sulusoglu and Cavusoglu [76] demonstrated that a positive correlation between these two reproductive metrics is not consistently observed. It is important to acknowledge that germination rate depends on the stage of flower development [77] and research revealed that pollen from hermaphroditic trees exhibited a lower germination rate compared to pollen from purely male trees [83]. In the present study, only two out of the three study sites exhibited a positive (although non-significant) correlation between pollen viability and pollen germination. At BY2, additional factors previously discussed but not quantitatively assessed may have influenced pollen germination. As germination was assessed only in 2022, our conclusions are primarily based on TTC viability, while acknowledging that germination assays may provide a more direct estimate of reproductive potential [62]. Overall, these findings highlight the multifactorial nature of pollen performance and its relevance for reproductive success in ash trees, underscoring the need for further research activities.

## 5. Conclusions

Pollen, as a key component of gene flow, did not show a consistent or pronounced decline in viability in response to ash dieback. Although severely affected trees tended to produce slightly less viable pollen, these effects were generally subtle and became evident only when accounting for hierarchical data structure in mixed-effect models. Overall, our results suggest that male ash trees largely retain the capacity to contribute viable pollen for fertilization across different levels of disease severity. In contrast, reproductive success

is likely to be constrained more strongly by reductions in flower and pollen production rather than by pollen viability itself. As ongoing ash decline is expected to substantially reduce overall pollen availability, further research should increasingly focus on quantitative aspects of pollen production and dispersal. In this context, studies on the aerobiology of ash pollen are essential for improving our understanding of gene flow and reproductive dynamics under progressing ash dieback.

**Author Contributions:** S.J.-O. was responsible for funding and the study design. Field work and data analyses was done by G.K.; writing, reviewing and proof-reading by G.K., L.B., A.-K.E. and S.J.-O. All authors have read and agreed to the published version of the manuscript.

**Funding:** The project receives funding via the Waldklimafonds (WKF) funded by the German Federal Ministry of Food and Agriculture (BMEL) and Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection (BMUV), administrated by the Agency for Renewable Resources (FNR) under grant agreement number 2219WK20H4.

**Data Availability Statement:** The data presented in this study are available from the corresponding author upon reasonable request.

**Acknowledgments:** We gratefully acknowledge the Bavarian State Forestry and the forest administration of each study site for permitting us to utilize their forests for our research endeavours. In addition, we thank Johanna Jetschni for her technical support in the field. During the preparation of the manuscript, the authors used DeepL Write (DeepL SE Version Next-Generation Language Model, Köln, Germany) and ChatGPT (OpenAI Version GPT-5.5, San Francisco, CA, USA), which are AI-assisted language tools designed to support text editing and language refinement. These tools were used solely to improve the language, readability, and clarity of certain passages. No AI tools were used for generating scientific content, data analysis, or data interpretation. The authors subsequently reviewed and edited the content as needed and take full responsibility for the content of the publication.

**Conflicts of Interest:** The authors declare that there are no conflicts of interest. There are only non-financial research interests, related directly or indirectly to this work submitted for publication. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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